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[*continued on p. iii of cover*

Geotropic Behaviour of Grass Nodes

N. ARSLAN[1] AND T. A. BENNET-CLARK

Received 6 August 1958

NORMAL geotropic curvatures are shown by young growing parts of plants. Certain mature nodes which have ceased growing, however, when subjected to a gravity stimulus such as placing in a horizontal position respond by an increase in size of the lower side (Sachs, 1865). This increase is irreversible and thus distinct from the pulvinar movements of leaf pulvini which are also influenced in certain cases by the gravitational field.

Gravity sensitive nodes are found in the Gramineae, Commelinaceae, and Caryophyllaceae as well as in other orders. The behaviour of such nodes has been studied by Sachs (loc. cit.), Elving (1884), Noll (1900), Rentschler (1929), and many others.

A reinvestigation of this behaviour is desirable as all these earlier studies were carried out before current hypotheses regarding influence of growth hormones became established. More recently, however, van Overbeek concluded that auxin accumulated on the lower sides of nodes of sugar cane when the canes were 'lodged' or laid horizontal (van Overbeek *et al.*, 1945). Even here this study by van Overbeek preceded the recent advances in technique which have shown the desirability of separating by chromatography growth factors which may be involved in plant response.

The present study is of the geo-reaction of two types of grass node. The motile tissue in some grasses consists of swollen and specialized leaf-base tissue which is sometimes described as a pulvinus (*Gelenk*). This seems an incorrect piece of terminology as pulvini proper of, for example, the Leguminosae, carry out reversible variation movements, whereas grass leaf nodal tissue undergoes an irreversible increase in size on geotropic stimulation. We will term the specialized grass leaf base a false-pulvinus to emphasize this physiological difference.

In other grasses the motile tissue is a portion of the stem immediately above the node.

MATERIAL AND METHODS

Most of the work was done with nodes of *Bromus sterilis* and *Triticum* (Wheat, variety Atle), grown in the garden of this Department. Second and third nodes from the inflorescence were used at a time just before opening of the spikelets.

In general, node preparations were made by cutting the stems 6 cm. above and below the node. These were fixed into 7 × 2 cm. specimen tubes between split corks with a filter-paper collar where the stem was gripped by the cork.

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The specimen tube was filled with water or a solution and the exposed cut end of the node preparation was vaselined. In certain experiments the inflorescence and leaves were left intact and in others one cut was made about 6 cm. below the third node and the other 6 cm. above the second or uppermost node, so that the preparation consisted of two nodes with the intervening internode intact and about 6 cm. of internode beyond the two nodes above and below.

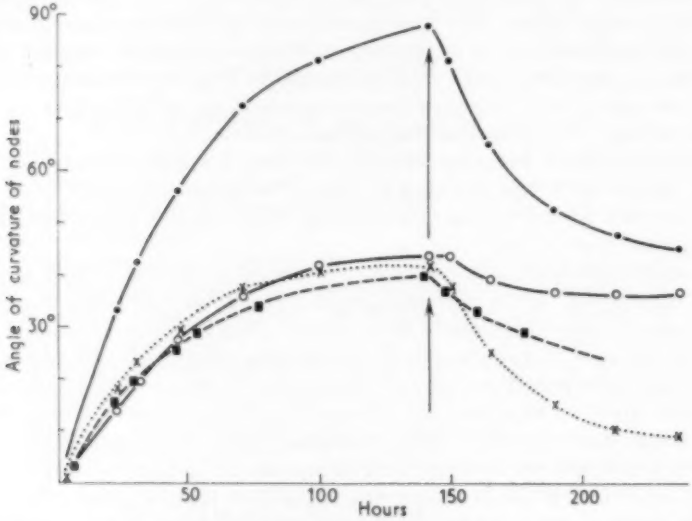

FIG. 1. Change in angle at nodes of shoots placed horizontally at zero hour. Upper node of a two-node preparation of *Bromus sterilis* (· · · x · · · ·), lower node of same preparation (—○—); single-node preparation of wheat var. Atle (- - ■ - -). The lower internode was returned to the vertical position at the arrow. The total angle to the horizontal of the upper internode of the two-node preparation is shown by the uppermost curve. All points represent means of 10 measurements.

The tubes holding the node preparations were fixed on to holders which could be easily placed either with stems and nodes vertical or horizontal. These were then placed in a paraffined wood chamber in which the atmosphere was nearly saturated. Light was excluded except when angular deflexions of the nodes were being measured. The deflexions of upper and lower nodes of a two-node preparation are termed θ_U and θ_L, and total deflexion θ_T is the sum of these.

Time course of movement with a two-node preparation. The typical behaviour of a preparation of two nodes of *Bromus sterilis* is shown by Fig. 1 which shows that on being placed horizontal upper and lower nodes both respond at nearly the same rate and response is almost complete after 100 hours. The period of rapid response is during the first 40 to 50 hours. It is noteworthy that the combined response of the two nodes raises the inflorescence

approximately into the vertical position. The data of Fig. 1 represent the mean of 15 node preparations. In occasional individual two-node preparations the lower node shows a somewhat greater response than 45° and this is sometimes accompanied by slight decrease of θ_U so that θ_T does not finally exceed 90°.

In the experiment of Fig. 1 after 142 hours the lowest section of the stem which had been placed horizontal was returned to the vertical position; this caused the uppermost section of internode to lie at an angle of 5° since θ_T had become 85°. θ_U decreased at about the same rate as it had increased after the first stimulus. This clearly straightened out the uppermost node, the

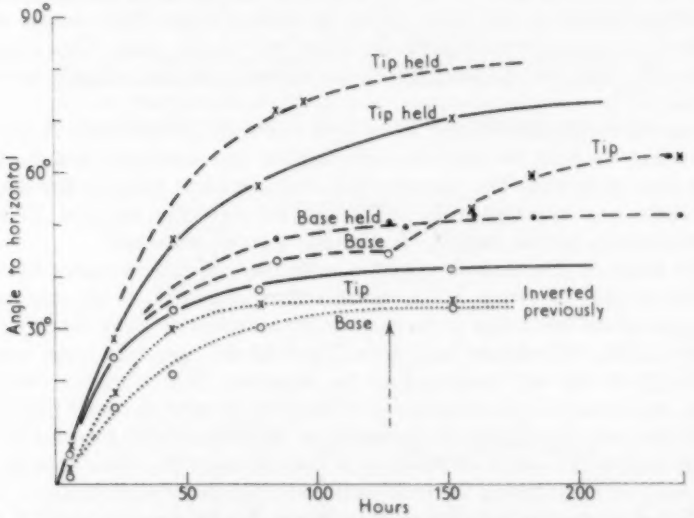

FIG. 2. Change in angle of single-node preparations of *Bromus sterilis* when lower (base-held) internode is held horizontal (o and •) and when the upper (tip-held) internode is held horizontal (—x—). Expt. M1 (—x—) and (—o—); Expt. M2, tip-held (- - x - -), base-held throughout (- - • - -), base-held for 128 hours and then reversed and tip-held (- - o - -, after reversal - - x - -). Expt. M3, material kept inverted for 20 hours before being placed with internodes horizontal (. . . .x. . . . and . . . .o. . . .). All points are means of 10 measurements.

straightening of the lower node was very slow and slight. This may be related to the fact that the middle section of internode sloped at about 45° compared with 5° for the upper section when the change in position through 90° was carried out.

Single-node response. This was examined with *Bromus sterilis* and *Triticum* Atle. The behaviour essentially resembles that of a single node of the two-node preparations. In Atle wheat the curvature in a typical experiment is shown also in Fig. 1 The deflexion attained a maximum of about 40°. This time course is nearly identical with that of *Bromus sterilis*. On replacing the lower internode (held in the tube) into the vertical position the bent node straightens out again slowly as shown in Fig. 1.

Effects of orientation on response. In the first experiments, of which representative examples have been described, the lower internode was held in the tube of water and was laid horizontal to give the stimulus. It was found that a different result was obtained when the upper internode was held in the tube and placed horizontal.

Results of typical experiments are shown in Fig. 2. Behaviour of base-held and tip-held single-node preparations cut on one occasion are shown as continuous lines in this figure. At first the rates of bending were similar but after 15–20 hours the divergence is very noticeable and the final angle of bending attained is about 50° in the base-held preparations. These represent the effects that are produced in Nature by wind and rain. The average angle of tip-held preparations was finally about 75° in this case. This value is frequently exceeded and in most of our experiments was actually between 85° and 100°.

In another experiment three series were examined (broken lines in Fig. 2). Some were tip-held but only two intermediate determinations and the final angle were measured. The rest were base-held, and one group of these base-held nodes were reversed at the 128th hour and were then tip-held. This reversal caused a further bending of the node through about 20°.

The effect of reversing the position as in this experiment shows that the normal 40°–50° curvature is due to some form of control of the amount of extension of the lower side of the node which is broken down by reversing the position of the horizontally held node. Tip-held and base-held nodes behave identically at the very beginning of the response. The difference develops when, as a result of the response, the length of internode raised physically above the node is respectively the apical or basal internode. In older terminology raising the apical internode to a position above the node reduces the geotonus, whereas raising the basal internode increases the geotonus.

Effect of orientation before stimulation. Since the different responses of tip- and base-held nodes show some superficial resemblance to the so-called tonic influence of gravity on normal orthotropic curvatures, it was thought that it would be well to carry out an experiment comparable to that of Frl. von Ubisch (1925). Two series of plants were kept for 18 hours in (*a*) normal position (*b*) inverse position. Single-node preparations of each were then made and were fixed as usual in the apparatus with either the tip held or base held.

Results are also shown in Fig. 2 by the dotted curves. Nodes from these inverted plants were less responsive and the difference between tip and base-held nodes was slight. This particular pair of experiments was not repeated. There were many repetitions of the other orientation experiments all giving similar results.

More recently Dr. Wilkins in this Department has shown that the difference in behaviour of tip and base-held nodes is conditioned by the amount of leaf-sheath tissue and if the leaf sheath is excised immediately above the node the difference disappears. The actual response is decreased but not eliminated when the leaf is removed immediately above the node.

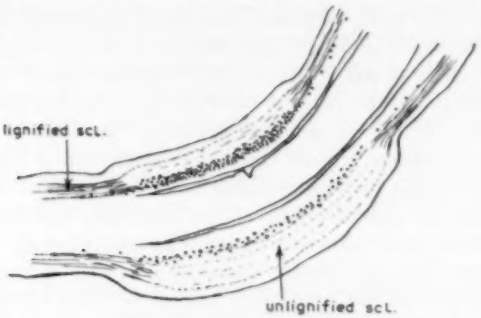

FIG. 3 (*a*). Camera lucida drawing of longitudinal section of a node after curvature to show the position of starch containing zones (dotted) and indicating diagrammatically the greater concentration of starch on the concave side. Note the kink in the intercalary meristematic part of the stem.

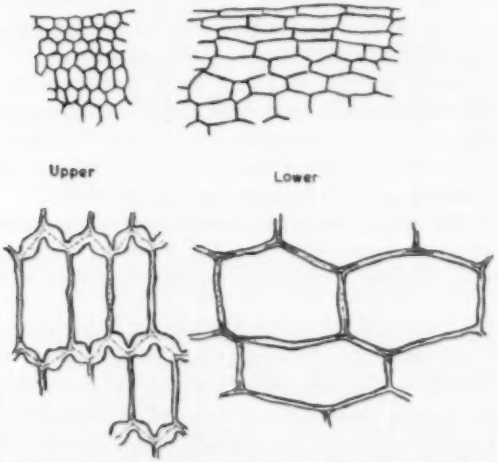

FIG. 3 (*b*). Camera lucida drawings of epidermal and specialized extension parenchyma from upper and lower sides of one bent node; and, at higher magnification, cells of the 4th row inwards, to show specialized periclinal walls.

Structural changes which produce bending. The 'motile tissue' which is responsible for the remarkable geo-response in wheat and in *Bromus* is a specialized swollen region at the base of the sheathing lower portion of the leaf. Certain features of this organ appear to be important. (1) Extremely massive columns of 'sclerenchyma' occur just outside the vascular bundles; although certain writers have called this tissue collenchyma, it seems right to term this tissue sclerenchyma since the cells of which it is composed are very long, sharply pointed cells essentially similar in shape to the sclerenchyma of the

rest of the leaf; they have, however, completely non-lignified walls and they have living contents which accumulate neutral red in their vacuoles and which can be readily plasmolysed. (2) Abundant 'statolith starch' is present in the parenchyma just inside the vascular bundles. These starch grains, it is readily seen, move freely to the physical lower side of the cells in which they lie. (3) The parenchymatous ground tissue and epidermis of the swollen nodal region consist of cells with remarkably thick longitudinal walls. It is also noteworthy that the sclerenchyma of the sheathing leaf base immediately above the swollen nodal portion is (as well known) strongly lignified as also is the epidermis. This lignification suddenly ceases as indicated in Fig. 3 to provide the unlignified and potentially extensible 'false pulvinus'.

When bending follows the geotropic stimulus, the changes are very characteristic and are indicated in Fig. 3. Quantitatively the cells of the lower convex side became some 2 to 3·5 times longer than they were. Actual measurements in a group of nodes which had bent through 42° showed that cells of the 4th row in from the epidermis had become 280 per cent. longer and correspondingly 25 per cent. narrower in the radial dimension. Cells of the corresponding layer on the concave side were 11 per cent. shorter longitudinally and non-significantly wider in the radial dimension.

The camera lucida tracings of Fig. 3 show that there is a characteristic pulling out of the thick longitudinal walls and it is tempting to postulate that the microfibrillar material of these walls is folded like the sides of a concertina. It is also quite clear that the cells of the non-lignified sclerenchyma at the same time clearly stretched, as their diameter is notably reduced.

The tissue of the upper side of the node becomes somewhat distorted and may sometimes show some wrinkling due to being compressed when the curvature due to geotropic stimulation is considerable as in the case of tip-held nodes.

The stem inside the cylindrical false-pulvinus is apparently passively bent. This is made clear when surgical operations are performed. If the lower half node is cut away, the upper half carries out a curvature but the enclosed stem remains almost straight. Operations of this type incidentally show that either an upper or a lower half node can respond when severed. They show also that response by the node occurs when the shoot is cut off immediately above and also when cut off immediately below the node. The response is therefore not dependent entirely on a transport of 'an auxin' into the false-pulvinus from its leaf or from the shoot below the node, though the difference in magnitude of response noted between tip-held and base-held node preparations suggests that some control of the magnitude of the response is exerted by material translocated from apexal or basal parts of the shoot into the node, the transport being influenced both by the gravitational field and the base-apex polarity of the plant.

Change in composition on extension. The large change in volume of the lower part of the false-pulvinus as a result of the gravity stimulus makes analysis a matter of special interest. In this study, material has been collected from the

two sides of the false-pulvinus at zero hour (i.e. before the stimulus of gravity) and at a number of time intervals after placing nodes horizontal.

The following data have been obtained: fresh weight, dry weight, reducing sugar content (using Hanes's modification of the Hagedorn-Jensen method (1929)), sucrose content, starch content, using the method of Pucher *et al.* (1948) and content of certain cell wall fractions by the methods of Jermyn and Isherwood (1956). Some further fractionation has also been made so that in addition to dry weight there are data of (*a*) 80 per cent. alcohol-soluble

TABLE I

Changes in the composition of two halves of geotropically stimulated nodes of Triticum (Atle)

Results are given as grams per 100 nodes: the samples analysed were respectively 100, 100, 87, and 97 nodes

	Hours after placing horizontal							
	0		18		46		90	
Total fresh wt.	5·29		5·01		5·68		6·20	
Total dry wt.	1·66		1·14		1·42		1·62	
Curvature of node degrees	0		22		55		46	
	left	right	upper	lower	upper	lower	upper	lower
Fresh wt.	2·64	2·65	2·12	2·89	2·15	3·53	2·56	3·64
Alcohol soluble material	0·30	0·31	0·18	0·25	0·19	0·30	0·24	0·34
Water soluble material ('pectin')	0·09	0·09	0·08	0·10	0·09	0·11	0·13	0·13
Water insoluble (cellulose, &c.)	0·41	0·41	0·31	0·23	0·30	0·37	0·37	0·42
Sugars (total conc. as mM.)	10·9	9·3	7·7	10·6	8·9	11·3	11·4	14·0

material (largely sugars and small molecules or ions capable of exercising osmotic effects; (*b*) alcohol-insoluble but water-soluble material which will include certain pectins and which in the following tables and figures is termed 'pectin'; (*c*) the water-insoluble material has been further fractionated by the Isherwood–Jermyn technique to yield fractions described as hemicelluloses *a* and *b* and α-cellulose.

The procedure employed was to place a fairly large number (between 1000 and 1500) node preparations in the horizontal position. At various times after zero-hour samples were taken for analysis and the leaf bases were split horizontally at the mid-line and the upper and lower halves separately sampled and analysed into fractions as indicated above. The unbent nodes, as at zero hour, were similarly treated and analyses of left and right halves made: differences between the halves at zero hour are an expression of variability of the material combined with the uncertainty introduced by the halving process which has to be judged by the eye. As there is considerable variation in the sizes of nodes, both in diameters and lengths, even when care is taken to select material for uniformity, it is most satisfactory to express changes in the various fractions as a percentage of the amount present on the upper side of the node as this *appears* to change little during the bending process.

The data on an absolute basis per 100 nodes are also available and are quoted in Table I.

The main trends are clearly shown for Expt. series A–E in Fig. 4. Length, fresh weight, and water content of the lower side of the nodes increase by about 70 to 90 per cent. as the angular movement advances during about 48 hours. This is as one expects. The data also show the relatively small changes in insoluble material and cellulose fractions, but considerable increase in alcohol soluble material and sugars in the lower half nodes.

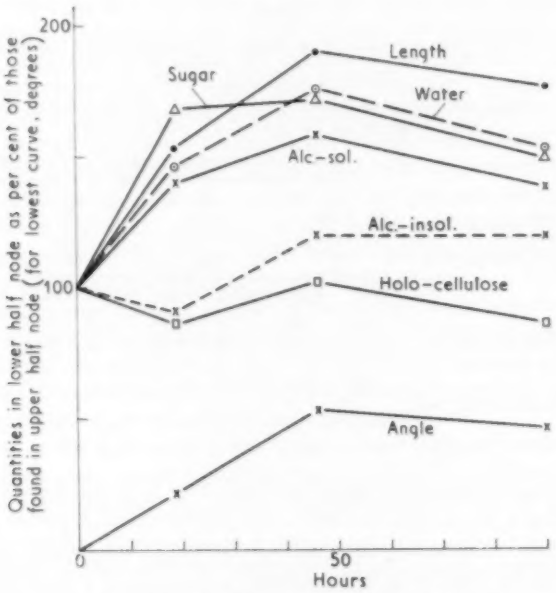

FIG. 4. Change in quantity of various constituents of the lower (convex) half of the leaf sheath at various hours after the single-node preparations of wheat var. Atle had been placed horizontal. Quantities are given as percentage difference from that shown by the concave upper half which does not change in length.

The conclusion is that bending is due to a cell extension not caused by additional wall formation or cellulose synthesis but by a stretching of existing walls. The data on the whole suggest that this stretching is very largely an osmotic effect brought about by a gain in alcohol-soluble substances which would presumably be osmotically active in the lower half node.

The data cannot at present completely exclude the possibility that a change in cell wall extensibility of the lower half node plays an important part in promoting this extension growth.

It will be noted that the curvature or bending of the single node had become complete at the 46th hour when a sample was taken for analysis. The next

sample taken 44 hours later was analysed with the object of finding out whether any further changes such as laying down of extra cellulose or consolidation of the extended cell walls had taken place. This had not occurred during this rather short period but straight anatomical study of considerably older bent nodes shows clearly that the cell walls do ultimately become thicker and after several days they become lignified.

Mechanism of induction of the geotropic response. Anatomical study shows very clearly that the geo-reaction is due to expansion of the parenchymatous ground tissue between the bundles of unlignified sclerenchyma of the node.

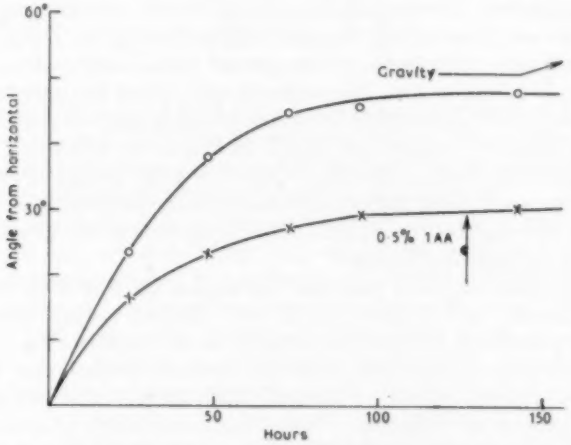

FIG. 5. Response induced by gravity in wheat compared with the response induced by 0·5 per cent. indolylacetic acid in lanolin emulsion.

These cells have thick (and presumably extensible) longitudinal walls. The gravitational stimulus causes these cells to enlarge in a longitudinal direction from 2- to 4-fold. This striking enlargement does not take place merely as a result of the passage of time by a slow growth process which becomes accelerated on the lower side of a node laid horizontal. The extra growth is *initiated* by laying the node horizontal. It is fashionable to explain growth as 'caused by' auxin and in the case of grass nodes we have shown that unilateral application of 3-indolylacetic acid in lanolin paste certainly does cause bending which superficially resembles the bending due to gravitational stimuli. This is indicated by the results of a typical experiment shown in Fig. 5 in which one can see that IAA applied unilaterally as 0·5 per cent. lanolin emulsion is only a little over half as effective as the geotropic stimulus. This concentration of IAA in lanolin is several times *more* effective than a geotropic stimulus in promoting coleoptile and hypocotyl curvatures as judged by the rate of bending. Maeda (1958) submitted his paper to this *Journal* since this present study was carried out and similarly finds that IAA

and also 2:4-D promote node extension. It is accordingly of some interest to find out firstly whether 3-indolylacetic acid or any other growth hormone is normally present in grass nodes or whether it is actually produced as a result of the stimulus.

The material used for this study consisted of *Zea mays* nodes as these are much larger than those of *Triticum* or *Bromus*. The anatomical mechanism is different in *Zea* as, following the stimulus, there is extension growth of a clearly specialized zone of the stem instead of growth of a leaf base. This specialized zone has ground tissue cells similar to those of *Triticum* leaf bases and the sclerenchymatous bundle sheaths are, as in the leaf base false-pulvini, unlignified. The specialized zone, moreover, contains very strikingly large amounts of starch in both *Zea* and *Triticum* (see Fig. 6). The gravitational stimulus induces enlargement of the ground tissue cells on the lower side by some 3- to 6-fold in *Zea*. The angle through which the internode is bent may, however, be quite small as the stems are fairly large in diameter. Nodes which had been left vertical and others stimulated by being laid horizontal were sampled for chromatographic analysis of their growth substances. In each case the nodes were halved; stimulated nodes yielded upper and lower halves and the controls left vertical yielded right-hand and left-hand halves which were supposedly identical.

Sampling methods were standard techniques as described by Bennet-Clark, Tambiah, and Kefford (1952) and Kefford (1954). Ethyl acetate extracts evaporated to low bulk were loaded on to Whatman No. 1 paper and the chromatograms developed with *iso*propanol-ammonia and bioassayed with wheat coleoptile segments. Results, which are not presented in detail as they are regarded as preliminary, reveal that considerable growth activity is present in two distinct regions of the chromatograms, one having an Rf considerably lower than that of IAA and a second with Rf slightly *larger* than that of IAA. Stimulated nodes showed apparently greater activity in this second zone especially in the upper half nodes, a result which is distinctly unexpected.

GENERAL DISCUSSION

The georeactions of the nodes of *Triticum*, *Hordeum*, and *Bromus* have been studied and shown to be due to similar responses of leaf base tissues. The response is *initiated* by the gravitational stimulus and consists of extension of specialized tissue which though capable of extension does not carry out this 'growth reaction' unless it receives the gravity stimulus. The growth reaction has been partly analysed. The increase in length, volume, and weight of the lower half of the false pulvinus is of the order of 60 to 100 per cent. This large increase is associated with a comparable (60 per cent.) increase in quantity of the alcohol-soluble constituents of the lower half. Total sugars of the lower half not only increase in *quantity* but this increase is greater than the increase in water content so that their *concentration* rises above that in the upper-half pulvinus.

By contrast the weight of 'wall material' does not appreciably change though there is a small reduction in wall weight in the first 20 hours followed by a later recovery and small gain in weight. The composition of the wall material did not appear to alter as shown in Table II.

TABLE II

Quantities of three fractions in the wall of the lower half node as percentage of total wall holocellulose

	Zero hour	18 hours	46 hours after placing horizontal
Hemicellulose A . .	37	33	29
„ B . .	20	21	18
α-cellulose . . .	43	45	54

The data of Tables I and II and Fig. 4 are certainly most simply explained on a basis of cell wall stretching by enhanced turgor, but changes in extensibility cannot be ruled out as the present analyses give no indication of presence of cross linkages on which extensibility must depend.

Tissue on the lower side of the geotropically stimulated node stretches about 300 per cent. without change in gross polysaccharide structure. Such stretching cannot be effected by application of a mechanical stretching force.

We have subjected nodes to bending moments on the same lines as those adopted by Noll (1895). He found that inflorescence stalks of *Agapanthus* bent, with a given moment, more when the geotropically stimulated lower side was being stretched than when the upper side was being stretched.

The reverse has been found in the grass nodes studied in this laboratory. The upper side of the stimulated node stretches more than the lower side. The situation is, however, very complex: the extensibility of the turgid tissue as a whole is conditioned only in part by the elasticity of the cell walls. It is also controlled by the cell turgor which if increased will tend to reduce or limit the deformation of cell shape which must occur when stretching occurs.

This will be more fully discussed in a later paper. It seems at present most likely that increase in turgor as revealed by the gain in alcohol-soluble material in the lower half nodes combined with an unfolding of folded long-chain molecules in the thick longitudinal walls of the node cortex are the essential parts of the mechanism. One might guess that such an unfolding would be somewhat comparable to the transition from α-keratin to β-keratin in the stretching of wool where the breakage of di-sulphide cross-linkages is a key part of the process. Neither the molecules nor their cross-linkages are yet certainly identified in these cell-walls.

LITERATURE CITED

BENNET-CLARK, T. A., TAMBIAH, M. S., and KEFFORD, N. P. (1952). Estimation of plant growth substances by partition chromatography. *J. Exp. Bot.* **9**, 343.

ELVING, F. (1884). Ueber das Verhalten der Grasknoten an Klinostaten. *Öfversicht Finska Vetensk. Soc. Förhand.* **26**, 107.

HANES, C. S. (1929). An application of the method of Hagedorn and Jensen to the determination of larger quantities of reducing sugars. *Biochem. J.* **23**, 99.

JERMYN, M. A., and ISHERWOOD, F. A. (1956). Changes in the cell wall of the pear during ripening. Ibid. **64**, 123.

KEFFORD, N. P. (1954). The growth substances separated from plant extracts by chromatography. I. *J. Exp. Bot.* **6**, 129.

MAEDA, E. (1958). The effects of growth regulators on the geotropism of the leaf sheath basal region in wheat. Ibid. **9**, 343.

NOLL, F. (1895). Ueber die Mechanik der Krummungsbewegungen der Gelenkpflanzen. *Flora*, **80**, 120.

—— (1900). Ueber Geotropismus. *J. wiss. Bot.* **34**, 457.

OVERBEEK, J. VAN, OLIVO, G. D., and SANTIAGO DE VASQUEZ, E. M. (1945). A rapid extraction method for free auxin and its application in geotropic reactions of bean seedlings and sugar cane nodes. *Bot. Gaz.* **106**, 440.

PUCHER, G. W., LEAVENWORTH, C. S., and VICKERY, H. B. (1948). Determination of starch in plant tissue. *Anal. Chem.* **20**, 850.

RENTSCHLER, H. (1929). Beiträge zur Kentniss der Wachstumskrummungen von Blattpolstern und Stengelknoten. *Bot. Arkiv.* **25**, 472.

SACHS, J. VON (1865). *Handbuch der experimental Physiologie der Pflanzen.* Leipzig.

UBISCH, G. VON (1925). Die Wirkungen der Schwerkraft auf Haupt- und Neben-wurzeln. *J. wiss. Bot.* **64**, 651.

The Effect of Indoleacetic Acid on Cell Wall Extensibility in *Avena* Coleoptiles

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SUMMARY

The extensibility of cell walls in segments of living *Avena* coleoptiles has been determined after treatment with IAA by the use of a microextensometer. It has been demonstrated that IAA has an effect both on the plastic and elastic extensibility, which is more marked if the segments are allowed to grow, except at temperatures nearing 0° C. The auxin-induced increase in extensibility is therefore not a direct effect on the wall and metabolic factors must be involved. The results are interpreted, along current lines of thought, as indicating effects on the linkage between protopectin or hemicellulose chains themselves and between these and the microfibrillar network of cellulose. The bearing of these interpretations on the normal growth process in cell walls is briefly discussed.

INTRODUCTION

THE discovery of growth-promoting substances in plants occurred at a time when interest was arising in the molecular structure of plant cell walls and at a period when the wall was widely considered to be stretched passively during growth. It was presumably this coincidence which led to the early preoccupation of workers in this field with the effects of these substances directly upon the wall, exemplified notably by the work of Heyn (1931). Subsequent demonstrations of effects of auxin on processes other than cell elongation changed the emphasis from the wall itself to the living contents of which it is the outer boundary. More recently, however, due in no small part to failure to find in the cytoplasm any auxin-controlled mechanisms which might itself explain the effects of auxin on growth, attention has again become centred on the wall particularly in attempts to explain auxin-controlled water uptake. It has been shown repeatedly that the sap obtained from auxin-treated tissue has a lower osmotic pressure than has that from auxin-free control tissue (Burström, 1942; van Overbeek, 1944; Levitt, 1947; Hackett, 1952; Brauner and Hasman, 1952). Auxin-induced water uptake can therefore be explained neither as a result of salt accumulation nor in terms of starch hydrolysis. It is therefore becoming generally assumed that the major growth-regulating effect of auxin is exercised on the wall. The mechanisms involved will be dealt with later; it may be noted at this point that a number of workers (Brauner and Hasman, 1949, 1952; Thimann, 1951; Burström, 1953; Levitt, 1953) have given firm support to Heyn's original conclusions. This support

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is derived, however, in the main from data largely concerned with other, if closely associated, problems and by rather indirect methods. Wall extensibility seems to have become a point of high significance in growth studies, and it is for this reason that the present attempt has been made to repeat and perhaps extend the work of Heyn using methods which, it is hoped, may be more critical.

When a wall is stretched under a steadily increasing load, the relation between the extension (ΔL) and the load (W) is usually of the form exemplified

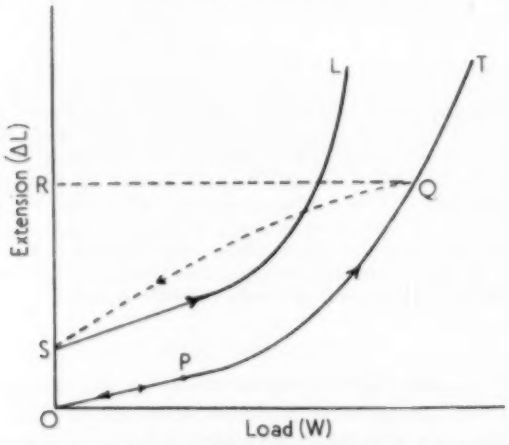

FIG. 1. Load/extension curve (diagrammatic). For explanation see text.

in Fig. 1. At low extensions (up to the point P in Fig. 1), ΔL varies linearly with W and the wall extension at any load is the same whether the load is increasing or decreasing. On removal of the load, the wall then recovers its original dimensions and the extension is elastic. It is over this range only that a *modulus of elasticity* can rigidly be defined. At points beyond P, whether the relationship between ΔL and W is still linear or not, the wall does not regain its original dimensions on the removal of the load. At the point Q, for instance, the total extension is OR, but on the release of the load a residual extension OS remains. This non-recoverable extensibility is referred to as plastic and is a measure of irreversible changes in structure which have occurred during stretching. The part SR of the extension is elastic and measures the reversible deformation of structure. Measurement of both OS and SR can therefore yield useful information concerning the molecular interactions involved in the stretching of a wall. It is particularly to be noted that when a wall is stretched from O to Q, is then allowed to return to S and is re-stretched, the second stretching SL does not follow the same path as the first even if the whole curve is transposed so that S and O coincide. In particular, the extensibility during a slight stretch from S is not the same as that involved in a slight stretch from Q, and the extensibilities do not necessarily arise from the same molecular

mechanism. The load/extension curve of a wall depends therefore upon the previous history of the wall.

It was this kind of consideration which led Heyn (1931, 1933) to determine the effect of auxin on the extensibility of cell walls. He concluded, from a long series of observations, that externally applied auxin markedly increased the plastic extensibility. At that time, knowledge of wall structure was not sufficiently advanced for him to provide a correct interpretation of this observation. Moreover, his results suffer from some uncertainty since he worked largely with plasmolysed tissue, and it is clear from what has been said above that the extensibilities he measured may have had little meaning in terms of the extensibilities of the turgid tissues before plasmolysis. His reasons for using plasmolysed tissue were, of course, sound, namely that, when a turgid cell is stressed longitudinally, changes in cell volume may induce strains in directions other than that of the applied stress. True moduli of elasticity may not then be derived.

In the work to be described, fully turgid tissue was nevertheless used, since, though interpretation of the extensibility in strict physical terms may then be uncertain, the conditions under which external stress is applied resemble more closely those operating during growth. The extensibility will usually be expressed in terms of the load required for a specific extension, which would appear to represent a parameter upon which the effect of IAA can be judged, and no attempt will be made to derive moduli of elasticity.

The procedure exemplified in Fig. 1 refers to the condition in which the load at any point of the curve is applied over a comparatively short period only. When, however, a constant load is applied to a wall for a prolonged period, the wall often elongates continuously. This is the phenomenon known as *creep*. It could be that, under the conditions of growth, creep is as important as—or even more important than—the elastic and plastic strains defined above. This will be considered in a later paper (Probine, 1959).

MATERIALS AND METHODS

Grains of *Avena* were dehusked, soaked in distilled water for 2 hours, and planted on microscope slides covered with wet filter-paper supported on glass rods in a glass half brick. This was covered by inverting over it a second half brick, and placed in a constant temperature room at 23°±0·5° C. in weak red light.

After 72 hours, coleoptiles of similar length (2·5–3·0 cm.) were selected, a 3 mm. length was removed from the tip of each and the next 10 mm. portion used in the various experimental treatments to be described. When these involved solutions, the coleoptile sections were floated on the liquid in petri-dishes; for treatment with lanolin paste, the sections were fixed upright in lanolin, with a blob on the upper end, and placed in a petri-dish lined with wet filter-paper. These operations were carried out either in diffuse daylight or in weak red light as occasion demanded. The material was then maintained in darkness at 23° C. except as specified below.

At various times during treatment the extensibility of these sections was determined in a microextensometer, the construction and operation of which have been described elsewhere (Spark, Darnborough, and Preston, 1958). Briefly this consists of a light beam, freely rotatable about a vertical axis near one end. The coleoptile is fixed between two clamps, one on the beam at a suitable distance from the axis, and the other on a fixed support. Movement of the beam then causes extension of the coleoptile and this extension is recorded as a change in the capacity of a condenser, one plate of which is

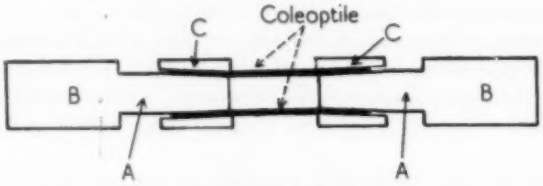

FIG. 2. Clamp used for attachment of segments to microextensometer.

attached to the movable end of the beam. The beam is caused to move by the application of a torque to the (vertical) spindle by the winding of a flat spiral spring, the inner end of which is attached by a link to the spindle. The winding mechanism is operated by a synchronous motor, so that the movement of the recorder chart gives a measure of the load applied, and the recorder therefore draws out a load/extension curve which can be calibrated. No attempt was made, however, to calibrate in terms of load/(wall area) so that the loads are given as arbitrary units per coleoptile.

The coleoptile sections were secured to the clamps as shown in Fig. 2. Each end of the coleoptile was threaded as a sleeve over a closely fitting metal rodlet *A* turned from the end of a thicker rod *B* which fitted in turn into the instrument clamps. A split metal collar *C* was then clamped over each coleoptile end, giving a secure fastening with a minimum of injury to the tissues. A coleoptile length of 2 mm. was left free between the clamps for extension. In the bulk of the determinations to be discussed, load was applied at a uniform rate which was kept constant throughout the series; and for simplicity the results are usually recorded as the load required to give exactly 10 per cent. extension. The instrument is such that this extension can be made to correspond to full-scale deflexion. On occasion, the load was released from that required to give 10 per cent. extension, at the same rate as it had been applied, so that a separation could be made between plastic and elastic extensibility. Each result quoted is the mean of 6 to 10 determinations.

The growth in length of the sections during treatment was clearly of critical importance, so that the length of an originally 10 mm. section was recorded for each section used in the microextensometer. Length changes during growth are recorded as 'elongation' to distinguish this from 'extension' in the microextensometer.

RESULTS

Sections immersed in water or IAA solutions (Figs. 3, 4, and 5). The extensibility data are recorded in Fig. 3. Sections prepared in diffuse daylight immersed in water showed a considerable initial *decrease* in extensibility during the first 2 hours of immersion, amounting to about 25 per cent. of the initial value. This was followed by a steadily *increasing* extensibility over the next 6 hours though the initial extensibility was not regained. An initial decrease in extensibility was also noted for sections in IAA at 1 mg./l. though less

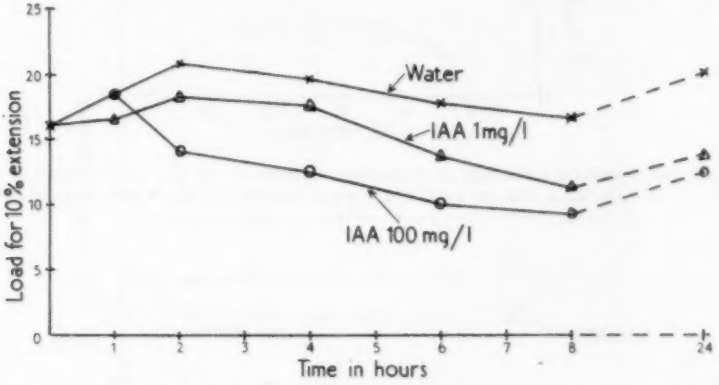

FIG. 3. Extensibility in relation to time of immersion in liquid at 23° C.; sections prepared in diffuse daylight.

marked than that in water alone. In IAA at 100 mg./l. this decrease occurred only during the first hour of treatment and in both solutions, as in water, there followed a steadily increasing extensibility. The extensibility remained throughout higher in IAA solutions than in water.

During the treatment period, the coleoptiles sections had increased in length to different extents depending on the treatment. When the load for 10 per cent. extension is plotted against the percentage elongation at the time of observation (Fig. 4) it is clear that there is a close relation between extensibility and elongation, especially in IAA solutions, in that as the sections grow they become progressively more extensible. For any elongation, however, the sections from the solution of higher concentration show the greater extensibility.

In a second series of determinations, the elastic and the plastic extensibilities were determined separately for sections in water and in IAA solutions at 1 mg./l. The results are recorded in Fig. 5. It should be noted that in this figure the ordinates represent percentage extensibility under a standard load. In water alone, the total extensibility over the first 4 hours is clearly related to changes in the plastic extensibility, the elastic extensibility remaining almost constant. After this period, the steady increase in total extensibility

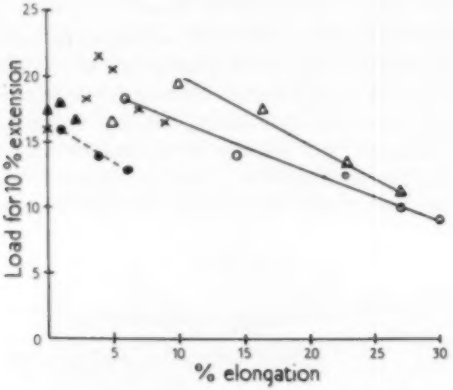

FIG. 4. Extensibility as a function of elongation. ×, water at 23° C.; —△—1 mg./l. IAA at 23° C.; —o— 100 mg./l. IAA at 23° C.; ▲ 1 mg./l. IAA at *c.* 0° C.; ● 100 mg./l. IAA at *c.* 0° C.

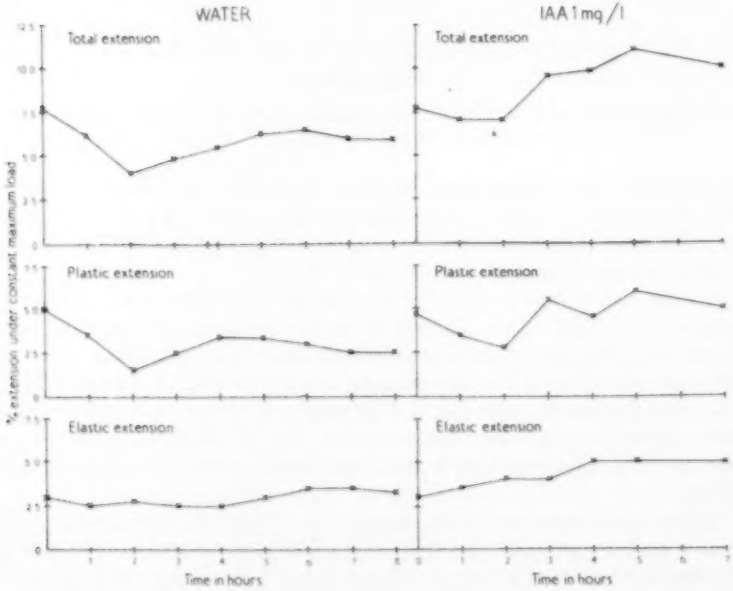

FIG. 5. Total extension, plastic extension and elastic extension for segments immersed in water (left hand) or IAA solution at 1 mg./l. (right hand).

seems to be due to an increase in the elastic component since the plastic extensibility steadily decreases. In the IAA solutions, the slight decrease in the extensibility during the first 2 hours is a consequence of the balancing of a much larger decrease in plastic extensibility by a concomitant increase in elastic extensibility. This latter increase seems to be conditioned by IAA. After the first 2 hours, the continuing increase in extensibility is due to an increase in both plastic and elastic extensibility.

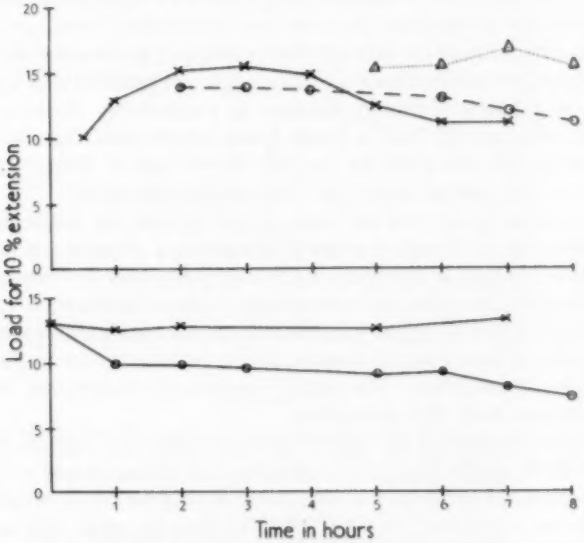

FIG. 6 (Upper). Extensibility after a period in darkness following illumination; —x— sections cut immediately after illumination, —o— sections cut after 2 hours of darkness, . . . Δ . . . sections cut after 5 hours of darkness.

FIG. 7 (Lower). Extensibility of segments prepared in weak red light after immersion in water (—x—) or in IAA solution at 50 mg./l. (—o—).

Thus the mechanical properties of coleoptile sections change in a somewhat complex way after immersion in these aqueous media. It is difficult to conceive of a mechanism which alone will involve a decrease in extensibility followed by an increase. It was therefore decided to investigate further the factors involved in the initial decrease. The decrease might clearly be associated with one of a number of factors, of which the three main ones seems to be (*a*) wound reactions consequent upon the preparation of the sections, (*b*) changed water relations induced by immersion, and (*c*) light effects, since the preparation of the sections were carried out in diffuse daylight. In order to separate this third factor from the other two, one batch of intact coleoptiles was illuminated for 20 minutes and then sections were prepared (i) immediately, (ii) after 2 hours in darkness, (iii) after 5 hours in darkness, and placed immediately in water. The total extensibilities of all three series were then

determined at various times after immersion and the results are presented in Fig. 6. A second batch was prepared in weak red light; some were then immersed in water and some in an IAA solution containing 50 mg./l., and the extensibility determination made as before. The results are presented in Fig. 7.

In the series prepared after daylight illumination (Fig. 6), those sections which were cut immediately after illumination show the initial decreased extensibility already noted. Sections cut 2 hours after cessation of illumination do not show this preliminary decrease but nevertheless have approximately the same extensibility as the first set when compared at the same time interval from the cessation of illumination. Thus it appears possible that light effects are involved in this preliminary decrease in extensibility. Sections cut at 5 hours from illumination have a much lower extensibility than those of the first two series but this could be due to a slower rate of elongation of intact coleoptiles or to a greater supply of wall-building materials.

Sections prepared in weak red light do not exhibit the initial decrease in extensibility (Fig. 7) though the rate of elongation is the same as for the sections prepared in diffuse daylight, and this confirms that the decrease is conditioned by light. In water, the extensibility remains constant over the whole period observed. It is therefore possible that the secondary increase in extensibility of sections immersed in water is also an indirect effect of illumination. In the IAA solution, there is a marked increase of extensibility which now proceeds immediately after immersion.

There can therefore be no doubt but that, under the conditions of these experiments, the application of IAA results in an increased wall extensibility. Since, however, the sections were elongating during the whole period, and the elongation was more rapid in IAA solutions than in water, this might have been only an indirect effect of IAA, operating through increased elongation, rather than a direct effect on the wall itself. The experiments which follow were designed to separate the indirect effect from any direct effect.

The effect of IAA on the extensibility of non-elongating sections

Elongation was arrested by each of three treatments: (*a*) by the use of low temperature, (*b*) by immersion at normal temperatures in approximately isotonic solutions, (*c*) by the withholding of water. The last of these three involved the application of IAA in lanolin paste.

(*a*) *Low temperature.* Sections were placed either in water or in solutions containing 1 mg/l. and 100 mg./l. IAA and kept in a refrigerator at 0° C. The sections in water and in the 1 mg./l. solutions showed only slight elongation in the first six hours and those in 100 mg./l. solutions extended to only 6 per cent. (Fig. 8 (*a*)). Only slight changes in extensibility were found (Fig. 8 (*b*)), the only marked trend being an increased extensibility of sections in the 100 mg./l. solutions, associated with elongation. It should, however, be noted that these sections at 0° C. are more extensible than are those of corresponding length at 23° C. (Fig. 4). This may imply that a wall 'hardening' process

occurs more slowly at 0° C. than it does at 23° C. and recalls the observation of Bonner (1936) that, at temperatures near freezing, elongation can occur with no concomitant wall synthesis.

(*b*) ***Immersion in isotonic solutions.*** Sections were immersed in 0·224M. KCl solutions, with and without IAA to the extent of 50 mg./l. Whether IAA was present or not, there was a marked increase in extensibility over the first 2 hours (Fig. 9); during this period the sections did not elongate. This could

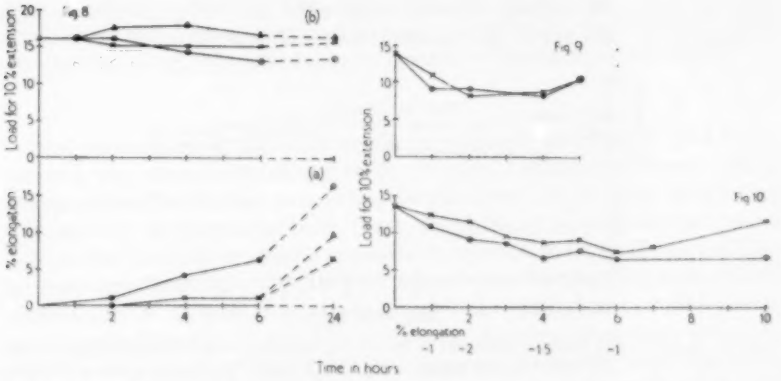

FIG. 8. Extensibility data for segments maintained in water (—x—) and IAA solutions at 1 mg./l. (—Δ—) and 100 mg./l. (—o—) at about 0° C. (*a*) Elongation of segments over a 24-hr. period. (*b*) Extensibility over the same period.

FIG. 9. Extensibility during immersion in isotonic KCl with (—□—) and without (—x—) IAA at 50 mg./l.

FIG. 10. As in Fig. 9, with KCl replaced by mannitol.

arise as a consequence of slight loss of turgor, and changes during this period should not perhaps be considered relevant. The more rapid increase in the IAA solution may be significant, though here again this could conceivably be associated with a more rapid loss of turgor induced by permeability changes. In the 2 to 4 hour period, the extensibility remains at about the same level in both treatments and during this time there is again no appreciable elongation. In the following hour, however, elongation is accompanied by a similar decrease in extensibility both in water and in IAA. This elongation may be due primarily to an uptake of ions, and leads to a return of the wall towards its original condition. IAA appears to have no effect under these conditions.

The experiment was therefore repeated using 0·4M. mannitol in place of KCl. This solution caused a preliminary slight shrinkage of sections during the first 3 hours, after which no further change in length occurred (Fig. 10). As with KCl, there was a steady increase in extensibility during the first hours as the sections were shrinking and this again is of greater extent in the presence of IAA. The difference between the extensibilities in water and IAA remains, however, during the 10 hours' duration of the experiment with no

complications due to uptake from the solution. The final decreased extensibility in the mannitol solution alone does not occur in the presence of IAA. It is to be noted that, since the shrinkages were the same at each point for sections in mannitol and in mannitol+IAA, it is unlikely that the different extensibilities were due to an IAA-accelerated water loss. It seems that here is evidence of a direct effect of IAA on wall extensibility.

(*c*) *The application of lanolin paste.* This is fully confirmed by further experiments in which IAA was applied in lanolin paste and the sections were not immersed in liquid; indirect effects through water and ion uptake and through elongation are then out of the question. Sections were prepared, and lanolin

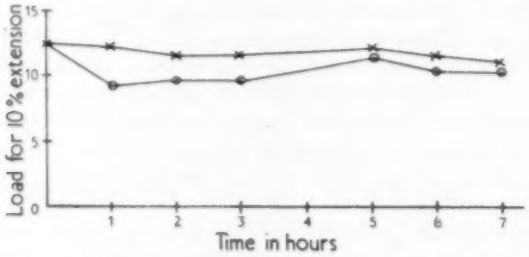

FIG. 11. Extensibility during treatment with IAA in lanolin. —x— lanolin alone; —o— lanolin containing 50 mg./l. IAA.

paste applied, in weak red light. Lanolin paste alone produced no appreciable change in wall extensibility over the 7 hours of observation (Fig. 11). Application of lanolin containing IAA produced a large increased extensibility in the first hour, remaining approximately constant and well above the extensibility of the control over the whole period.

TABLE I

Percentage extension of coleoptiles under the same standard load

Treatment	Total	Elastic	Plastic	Plastic as % total
Original coleoptile . . .	10·3	9·3	1·0	9·3
Lanolin, treated 1 hr. . .	9·8	8·1	1·7	17·4
Lanolin and IAA, treated 1 hr. .	11·9	9·7	2·2	18·8

When this experiment was repeated with sections cut in diffuse daylight, some significant differences were observed. There was no initial decreased extensibility such as was observed for similar sections placed in water. Thus light exerts its influence only when water is available and the section is elongating. Further, however, the increase in extensibility on the application of IAA was of smaller extent than for sections cut in weak read light. Thus it appears that a light pretreatment reduces the subsequent response to IAA as far as wall extensibility goes.

If the results of lanolin-treated sections prepared in weak red light are compared with similar sections immersed in water (Figs. 7 and 11) several further

points emerge. The same initial increase in extensibility occurs in each case, though one set of sections has not elongated while the other has elongated by some 6 per cent. in the first hour. In both cases the extensibility thereafter remains constant at the same level although there are considerable differences in elongation.

Finally, this clear-cut effect of IAA applied in lanolin led to experiments in which the plastic and elastic extensibilities were measured separately. These are presented in Table I as the percentage extension under the same load after 1 hour's treatment. The indications are clearly that IAA causes a marked increase both in elastic and plastic extensibility over controls treated with lanolin alone. The effect is more marked with plastic extensibility.

DISCUSSION

The present results therefore amply demonstrate an effect of IAA in increasing the extensibility of the walls of living turgid parenchyma cells of *Avena* coleoptiles whether or not the cells are allowed to elongate. In this they confirm fully the opinion of Heyn (1931, 1933), based on plasmolysed tissues, except that the effect on elastic extensibility appears to be more marked than he supposed. Externally applied IAA affects both the elastic and the plastic extensibility of the wall, though not always to the same extent or even always in the same direction. It could be suggested, therefore, that there are in the wall two components at least which are separately affected by IAA. This effect cannot be due to auxin-induced changes in wall composition for Burström (1958) has shown that such changes as do occur are too small to be of significance. The lack of any effect either on the walls of dead cells, or on living cells at 0° C., suggests that the action of IAA is not direct but is conditioned by intervening metabolic factors. This is in complete harmony with present ideas on the mode of action of growth substances, and will be discussed again below.

It is now clearly recognized that the walls of cells of *Avena* coleoptiles—as of all other plant cells—can be considered as a two-phase system. The greater area of the wall consists of a mesh of microfibrils of the order of 100 Å diameter, somewhat randomly disposed with a tendency towards transverse orientation, and lying in a highly hydrated matrix of incrusting substances. The microfibrillar component occupies only a small fraction of the total wall volume which may be as low as 2·5 per cent. (Frey-Wyssling, 1952). A further 5 per cent. of the wall volume is occupied by (dry) hemicellulose, pectins, &c., and the remainder is water. The whole of this loose structure is reported to extend as the cell grows (Castle, 1955; Scott *et al.*, 1956; Wardrop, 1955, 1956; Wilson, 1957; Gorham and Colvin, 1957), and it is clearly in terms of such a structure that the above results will need to be interpreted.

The question naturally arises if the elastic component of wall extensibility is to be attributed to one wall fraction—the microfibrils—and the plastic extensibility to the other—the incrusting substances. It seems reasonable to conclude that such a rigid separation cannot be made.

The microfibrils consist of a central crystalline core, occasionally interrupted, which may be 20–50 Å in diameter, with a covering of molecular chains more loosely arranged of the order of 20 Å or so thick (Preston and Cronshaw, 1958; Preston, 1959). Since these microfibrils tend to be oriented transversely, moderate longitudinal extension of the walls involves a bending and twisting of microfibrils with internal readjustments which may be small. These extensions could then be elastic. Occasional excessive bending or twisting could be irreversible, however, leading to some plastic extension. It is well known that the cellulose component of mature cell walls can extend both elastically and plastically, depending on the degree of extension (e.g. Spark, Darnborough, and Preston, 1958; Spark and Preston, 1959) and the same has recently been shown true of growing walls (Probine, 1959).

The microfibrils are embedded in, and possibly attached (if somewhat loosely) to, the incrusting materials which are said to consist in the main of pectic substances and hemicelluloses. These are associated with anions (e.g. Ca^{++}, Mg^{++}) and cations (e.g. HPO_4^{--}), and a recent review of the evidence available has led Henglein (1958) to a schematic representation of the condition of protopectin and its associations in the wall. Bishop, Bayley, and Setterfield (1958) have recently reported that the cell walls in *Avena* coleoptiles contain only 0·3 per cent. of pectic compounds (on a dry weight basis) as against 51 per cent. of hemicelluloses. They therefore suggest that the physical properties will depend on the hemicelluloses and not on the pectins. The following discussion is presented in terms of pectins, however, since these may be of greater importance to cells generally. Most of it would apply, moreover, also to the hemicelluloses except the intervention of the particular enzymes system mentioned. The representation by Henglein is presented in a modified form in Fig. 12, drawn roughly to scale. For clearness of figure, however, chains of protopectin (full lines) are drawn much too thick and much too infrequent and the bonds uniting them much too long. This figure makes it clear that two neighbouring cellulose microfibrils are held together in the wall by a profuse network of interconnected polyuronide chains. In order to separate them—and therefore allow wall extension—either the meshes of this network must be deformed by rotation about the linking bonds which would lead to elastic extension, or bonds must be broken and mutual displacement of chain molecules occur, leading to plastic extension of the pectic framework. In either case the cellulose component could be deformed elastically, but in the latter case the microfibrils might be unable to return to the initial configuration on the release of stress, owing to the deformation of the intervening pectic network.

On the grounds both of the relative amounts of cellulose and incrusting substances and of the relative dispositions of these two components exemplified in Fig. 12, it seems that changes in extensibility of the cell wall are to be explained more readily by reference to pectin than to cellulose. This is in complete harmony with some current views on the biochemistry of auxin-regulated wall growth. It was suggested many years ago that it is the pectins

and related substances rather than cellulose which strengthen the cell wall in the longitudinal direction (Kerr, 1951) and this has recently received new expression in the work of Ordin and Bonner (1957). These workers have shown that esterification of pectic acid by methyl-derived carbon is an auxin-controlled process. It could be that methylation of carboxyl groups on adjacent pectic molecules, under the control of auxin, involves the splitting of anhydride or calcium bridges which contribute to the mechanical properties of the wall. As already pointed out by Bennet-Clark (1955), the extensibility of the wall would be expected to increase if linkages between pectic chains

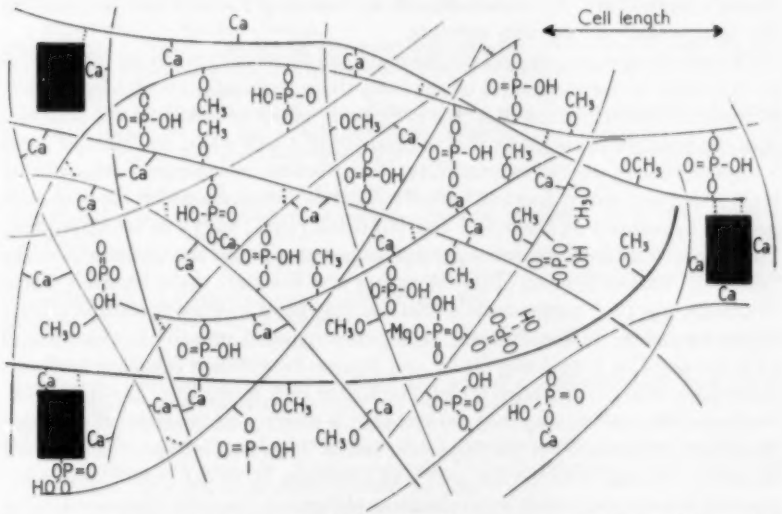

FIG. 12. Diagrammatic representation of spatial relations between cellulose and incrusting substances; approximately to scale. The wall is viewed in longitudinal radial section; the black rectangles represent cellulose microfibrils in cross section and the full lines molecular chains of pectin. Dotted lines represent hydrogen bonds.

through, for example, multivalent cations were replaced by van der Waals forces consequent upon esterification. Hydrogen bonding between free carboxyl groups would provide an intermediate condition. Methylation of pectic compounds is known to be under the control of pectin methylesterase (PME) and Glasziou and Inglis (1958) have proposed that an auxin-controlled binding of PME, immobilizing the enzyme and therefore reducing its activity, would favour an increased degree of methylation of the pectic components in the wall. This would lead to an increase in wall extensibility. Ordin and Bonner (1957), however, do not find any indication of a change in PME activity on treatment of *Avena* coleoptiles with auxin.

Extensibility of cell walls may depend, therefore, on a complex of factors the relative importance of which varies with degree of extension. At low extensions (say up to 1 per cent.) the extension is elastic and depends upon slight

deformations both in the protopectin matrix and in the microfibrils. Somewhat higher extensions have a plastic component, arising from slip between protopectin chains (due to breakage of hydrogen and other bonds) which prevents the return of the elastically deformed microfibrils, and such meshes of the protopectin network as are elastically deformed, to the initial configuration on the removal of stress. At still higher extensions, elastic deformation may perhaps be attributed solely to the microfibrils, which may also be deformed plastically. At all extensions, auxin could then increase both the plastic and the elastic extensibility of the protopectin by loosening inter-chain linkages, and elastic extensibility of the microfibrils, by reducing the restraint imposed by the intervening protopectin network.

The effects of auxin on wall extensibility can therefore readily be interpreted on the basis of the mechanics of the long-chain molecules involved. It is not, however, thereby proved that this is the only effect of auxin on the wall and it is not possible to conclude that the growth of a wall—and therefore of a cell—depends solely on wall extensibility. Nevertheless, whether wall extensibility is regarded as of overriding and overall importance, as envisaged in the multi-net growth hypothesis of Roelofsen and Houwink (1951, 1953), or whether other factors are considered to be of greater importance, as in the variations on the theme of 'mosaic growth (Frey-Wyssling and Stecher, 1951; Stecher, 1952; Wardrop, 1955), it seems certain that the first stage in growth must involve a slight extension. If it is the case, as again suggested recently in the work of O'Kelly and Can (1953) and Ordin and Bonner (1957), that cellulose synthesis keeps pace with growth, then it could be that at each moment of growth the extension of the wall during the next moment is elastic and the effect of auxin on the elastic extensibility is the important factor. This would imply the necessity for only a minimal interference with wall structure. If, on the other hand, cellulose synthesis is completely separable from elongation, then the effects of auxins, involving plastic extensions, must be more far-reaching. On this point, it was shown long ago by Bonner (1936) that coleoptile segments can elongate at low temperatures with almost no wall synthesis. This does not, however, mean that in normal growth wall synthesis and elongation are two separate processes; indeed the lack of any auxin effect at 0° C. suggests that they are not.

LITERATURE CITED

BENNET-CLARK, T. A. (1955). In *Chemistry and mode of action of Plant growth substances*. ed. Wain and Wightman.

BISHOP, C. T., BAYLEY, S. T., and SETTERFIELD, G. (1958). Chemical constitution of the primary cell walls of *Avena* coleoptiles. *Plant Physiol.* **33**, 283.

BONNER, J. (1936). Zum Mechanismus der Zellstreckung auf Grund der Micellarlehre. *J. Wiss. Bot.* **82**, 377.

BRAUNER, L., and HASMAN, M. (1949). Über den Mechanismus der Heteroauxin-Wirkung aud die Wasseraufnahme von pflanzlichem Speichergewebe. *Istambul Univ. Tip. Fac. Mac.* **12**, 57.

—— (1952). Weitere Unterauchungen über den Wirkungsmechanismus des Heteroauxins bei der Wasseraufnahme von Pflanzenparenchymen. *Protoplasma*, **41**, 302.

BURSTRÖM, H. (1942). Die osmotischen Verhältnisse während des Streckungswachstums der Wurzel. *Landbruckshogsk. Ann.* **10**, 1.

BURSTRÖM, H. (1953). Growth and water absorption of *Helianthus* tuber tissue. *Physiol. Plantarum*, **6**, 685.
—— (1958). The influence of growth regulators on the composition of the cell wall. *Kungl. Fysiograf. Sallsk. i Lund Förhandl.* **28**, 54.
CASTLE, E. S. (1955). *Proc. Nat. Acad. Sci. U.S.* **41**, 197.
FREY-WYSSLING, A. (1952). Growth of plant cell walls. *Symposia Soc. Exp. Bot.* **6**, 320.
—— and STECHER, H. (1951). Das Flächenwachstum der pflanzlichen Zellwände. *Experientia*, **7**, 420.
GLASZIOU, K. T., and INGLIS, S. D. (1958). The effect of auxins on the binding of pectin methylesterase to cell walls. *Austral. J. Biol. Sci.* **11**, 127.
GORHAM, P. R., and COLVIN, J. R. (1957). Cellulose deposition in elongating epidermal cells of *Avena* coleoptiles. *Exp. Cell. Res.* **13**, 187.
HENGLEIN, F. A. (1958). Die Uron- und Polyuronsäuren. In *Handbuch der Pflanzenphysiologie* VI. Springer-Verlag.
HEYN, A. N. J. (1931). Der Mechanismus der Zellstreckung. *Rec. trav. bot Néerl.* **28**, 113.
—— (1933). Further investigations on the mechanism of cell elongation and the properties of the cell wall. *Protoplasma*, **19**, 78.
KERR, T. (1951). Growth and structure of the primary wall. In *Plant Growth substances*, ed. F. Skoog. Pp. 37. Univ. Wisconsin Press, Madison.
LEVITT, J. (1947). The thermodynamics of active (non-osmotic) water absorption. *Plant Physiol.* **22**, 574.
ORDIN, L., and BONNER, J. (1957). *Plant Physiol.* **32**, 212.
OVERBEEK, J. VAN (1944). Growth-regulating substances of plants. *Ann. Rev. Biochem.* **13**, 631.
PRESTON, R. D. (1959). Wall organization in plant cells. *Int. Rev. Cytol.* **8**. In the press.
—— and CRONSHAW, J. (1958). Constitution of the fibrillar and non-fibrillar components of the walls of *Valonia ventricosa*. *Nature*, **181**, 248.
PROBINE, M. C. (1959). To be published.
ROELOFSEN, P. A., and HOUWINK, A. L. (1951). Cell wall structure of staminal hairs of *Tradescantia*. *Protoplasma*, **40**, 1.
—— (1953). Architecture and growth of the primary cell wall in some plant hairs and in the *Phycomyces* sporangiophore. *Acta Bot. Néerl.* **2**, 218.
SCOTT, F. M., HAMNER, K. C., BAKER, E., and BOWLER, E. (1956). *Amer. J. Bot.* **43**, 313.
SPARK, L. C., DARNBOROUGH, G., and PRESTON, R. D. (1958). Structure and mechanical properties of vegetable fibres. II. A micro-extensometer for the automatic recording of load/extension curves for single fibres. *J. Text. Inst.* **49**, T 309.
—— and PRESTON, R. D. (1959). To be published.
STECHER, H. (1952). Über das Flächenwachstum der pflanzlichen Zellwände. *Mikroskopie*, **7**, 30.
THIMANN, K. V. (1951). *Growth Symposium*, **10**, 5.
WARDROP, A. B. (1955). The mechanism of surface growth in parenchyma of *Avena* coleoptiles. *Australia J. Bot.* **3**, 137.
—— (1956). The mechanism of surface growth in the parenchyma of *Avena* coleoptiles. *Biochim. Biophys. Acta*, **21**, 200.
—— (1956). The nature of surface growth in plant cells. *Australian J. Bot.* **4**, 193.
WILSON, K. (1957). Extension growth in primary cell walls with special reference to *Elodea canadencis*. *Ann. Bot.* N.S. **21**, 1.

Photoperiodism in the Light-inhibited Seed of *Nemophila insignis*

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INTRODUCTION

FOLLOWING the discovery by Borthwick and co-workers (1952) that the action spectrum for germination of certain light-requiring seeds is the same as that for photoperiodic control of flowering, it was shown by Isikawa (1954) and Black and Wareing (1954) that the germination of seeds of a number of species is under direct photoperiodic control.

A detailed investigation of germination in *Betula pubescens* (Black and Wareing, 1955) revealed many close analogies between the photoperiodic behaviour of seeds and dormant buds of this species. Exposure of birch seed to long daily photoperiods promotes the germination of approximately 90 per cent. of the seeds as opposed to only approximately 30 per cent. germination under short-day conditions. Thus, birch can be regarded as a 'long-day' seed.

Studies by Isikawa (loc. cit.) on the germination of seeds of several herbaceous species indicated that certain seeds give higher germination percentages in short days than in long days. However, no intensive investigation has been made of photoperiodism in 'short-day' seeds. Therefore, as a corollary to our work with the light-requiring, 'long-day' seed of *Betula pubescens*, a study has been made, with special reference to the effect of the photoperiodic régime, of germination in *Nemophila*, which is known to be a light-inhibited seed (Lehmann, 1909).

MATERIAL AND METHODS

General germination techniques. Seed of *Nemophila insignis* was obtained from commercial sources.

In testing the effect of a particular treatment, seeds were placed in petri-dishes on filter-paper saturated with distilled water. At least 100 seeds were used for each treatment and because of their small size (about 3 mm. long) these were sown in one dish.

Seeds were treated in thermostatically controlled light cabinets. For photoperiodic régimes, the dishes were wrapped in black paper to provide a dark period. As the source of white light, 'daylight' fluorescent tubes were

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used, the intensity at seed level being 4,400 ergs./cm.²/sec. 'Monochromatic' light was obtained from coloured fluorescent tubes in conjunction with coloured perspex or from Schott line interference filters. Further details of these and of the infra-red sources are given below. Light intensities were measured by a barrier-layer photocell which had been calibrated against a thermopile for each type of light source. The thermopile was first calibrated against a standard tungsten-filament lamp. Infra-red radiation was measured by a photocell (G.E.C. type C.M.U.6) having a caesium-silver oxide cathode.

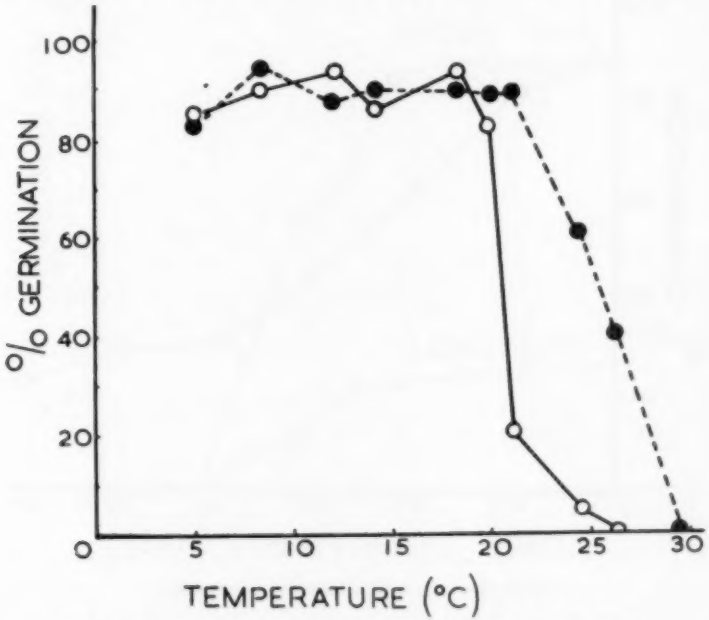

FIG. 1. The effect of temperature on germination in light and darkness. (——○——) in light, (-----●-----) in darkness.

EXPERIMENTAL RESULTS

The influence of light and temperature

Initial experiments on the effect of light gave extremely diverse results which were found attributable to small temperature variations in the region of 21° C. A series of experiments carried out to study the interaction between the inhibitory effect of light and temperature showed that at temperatures below 21° C. there is little difference between the germination in light and darkness. To obtain the results shown in Fig. 1 seeds were kept in continuous light or darkness until no further germination took place (about 6 days). It is clear that germination is inhibited above 22° C., even in darkness, and at 28–29° C. germination is completely suppressed.

Between 20° and 25° C. germination is inhibited by light, the effect being most marked at 21°–22° C. Subsequent experiments were, therefore, carried out at 21°–22° C., except where otherwise stated.

The effect of daylength

The above experiment shows that continuous light inhibits germination, an effect most apparent at 21°–22° C. To determine whether there is a differential

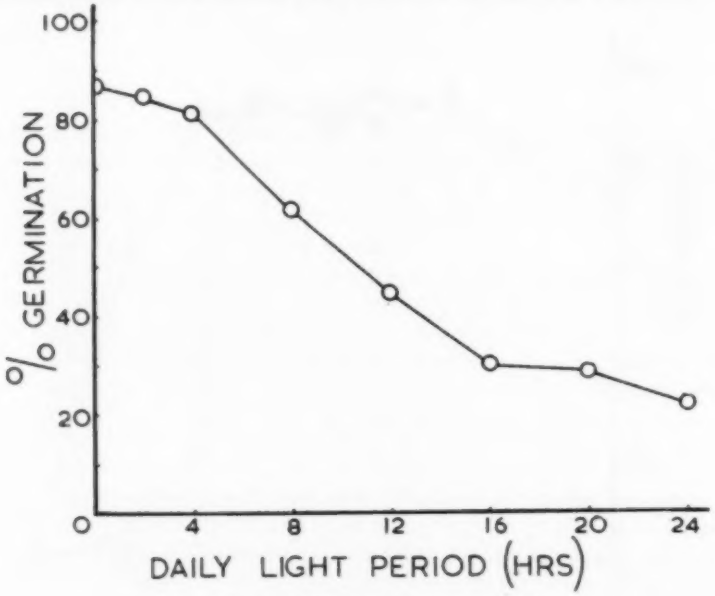

FIG. 2. The effect of daylength on germination.

effect of various photoperiods, seeds were exposed to daily light periods of 4, 8, 12, 16, 20, and 24 hours using 'daylight' fluorescent tubes at 4,400 ergs/cm.^{2}/sec. at the above temperature. The treatments were continued for 7 days; it was observed that no further germination occurred after 4 days. The final germination percentages are shown in Fig. 2. The effect of daylength is distinct and these results have been repeated numerous times. In all cases, seeds exposed to very short daily photoperiods give a germination percentage close to those in darkness, but as the daily light period increases there is a corresponding decrease in the percentage of seeds which germinate. Thus, there is no clearly defined 'critical' daylength for any given population of seeds. Evidence that these are true photoperiodic effects is given by later experiments, but it seems likely from this experiment that the total duration of light or darkness is not the determining factor, since the germination figures remain constant even with continued treatment (Fig. 3).

Relative roles of the light and dark periods

In both long-day and short-day species the flowering response is determined primarily by the absolute length of the dark period, and also, in some species, of the light period, e.g. Biloxi soybean (Hamner, 1940).

In seed of *Nemophila* the question now arises as to whether germination is inhibited in long days by the excessive length of the light period or by the

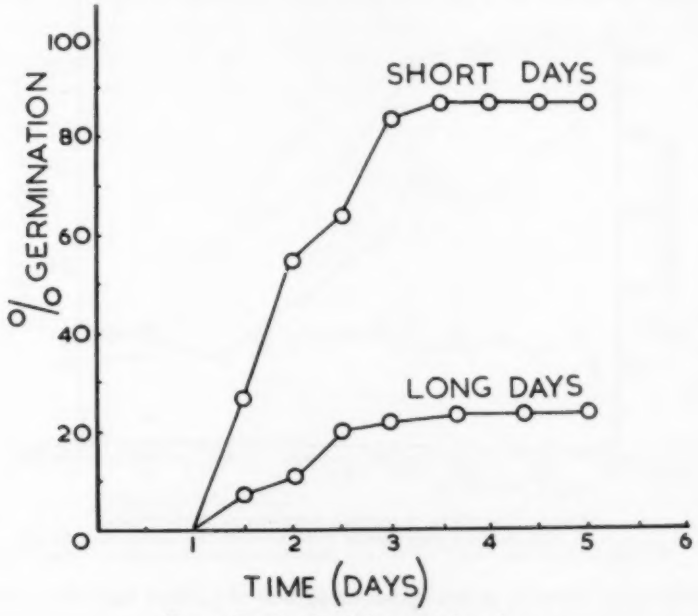

FIG. 3. Germination in long and short days.

shortness of the accompanying dark period. Experiments described below were carried out to investigate this problem.

Experiment 1. In this experiment the effects of various durations of light period in association with constant dark periods of 4 or 20 hours were investigated. The treatments shown in Fig. 4 were continued until no further germination took place (5 days). The curves show that even short light periods inhibit germination but only when associated with a short dark period. On the other hand, moderately long photoperiods (of, say, 20 to 24 hours) fail to inhibit germination if the accompanying dark period is also long. It would, therefore, appear that the absolute length of the dark period is of prime importance, although an effect of very long photoperiods can be seen, even when the latter accompanies long dark periods.

Experiment 2. The effects of exposing seeds to different lengths of dark period using photoperiods of constant duration were studied in the present

experiment. The results shown in Fig. 5 might have been predicted from the results of the preceding experiment. Again it is clear that the absolute length of the dark period is determinative, and that as this increases there is a corresponding increase in the percentage of seeds which germinates.

Experiment 3. From the above results it seems unlikely that germination is determined simply by the total duration of light or dark to which the seeds are exposed. However, the present experiment was performed to verify this.

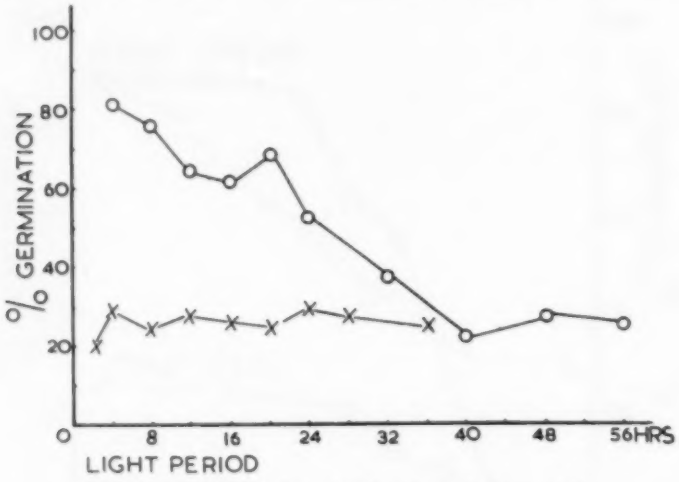

FIG. 4. The effect of varying durations of photoperiod (——o——) with 20-hour dark period; (——x——) with 4-hour dark period.

Seeds were exposed to five cycles composed of 4 hours light and 4 hours darkness. They were then transferred into continuous light. Simultaneously a second group of seeds was submitted to a cycle consisting of 20 hours light followed by 20 hours darkness, these also being afterwards transferred to continuous illumination. The germination percentages, determined after 4 days, are given in Table I. These results demonstrate that although the seeds were exposed to the same total amounts of light and darkness, there are very marked differences in response. Apart from this confirmation that the total durations of the periods of light and darkness are not decisive, it is apparent also that one long dark period alone suffices to allow of the germination of a large percentage of seeds.

The effect of a 'light-break'

It is well known that the effect of a 'light-break' during a long dark period profoundly modifies the response of both long-day and short-day herbaceous species. To investigate the effect of a 'light-break' on the germination of *Nemophila*, seeds were exposed at 21° C. to the light régimes shown in Table

II. The experiment was continued for 4 days when the percentage germination under each treatment was determined. It is clear from the results that an interruption of the dark period by a 'light-break' has a marked effect on germination. This is in agreement with results obtained for seed of *Betula*

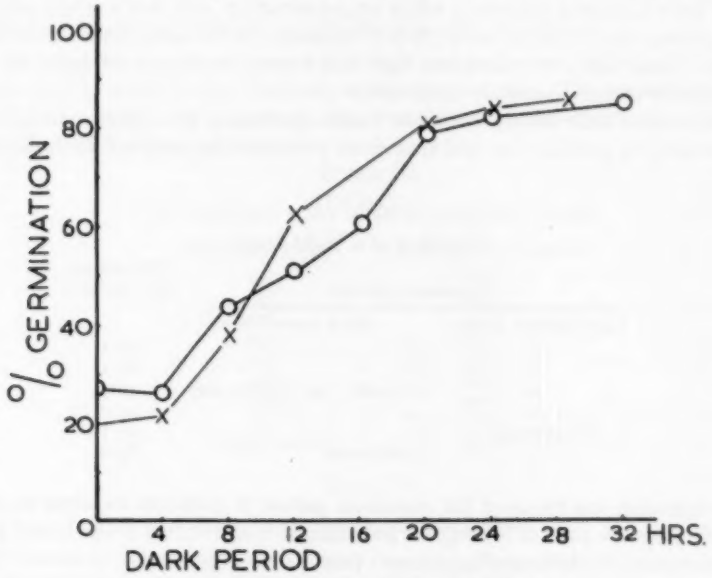

FIG. 5. The effect of varying durations of dark period (——o——) with 20-hour photoperiod, (——x——) with 4-hour photoperiod.

TABLE I

Germination of seeds of Nemophila exposed to the same total amounts of light and darkness but given different cycles

Treatment	Percentage germination
5 cycles. 4 hrs. light–4 hrs. dark . .	33·3
1 cycle. 20 hrs. light–20 hrs. dark . .	70·7
Continuous light	28·8
Continuous darkness	86·7

pubescens (Black and Wareing, loc. cit.) and presumably corresponds to the 'light-break' effects in the dormancy of *Fagus* buds (Wareing, 1953) and in the flowering response of many herbaceous species.

The effect of single light and dark periods

Approximately 75 per cent. of the seeds fail to germinate when exposed continuously to light at 21°–22° C. Moreover, these inhibited seeds do not germinate even when afterwards transferred to darkness at the same temperature; they are thus said to be light-hard.

Experiments were performed in order to determine the minimum period of illumination, from time of sowing, necessary to give marked inhibition and to produce this condition. It was found that single-light periods of up to 24 hours duration have little inhibitory effect, that light periods exceeding this have a marked inhibitory effect on germination, and that a single period of 32 hours produces the same order of inhibition as does continuous illumination. Thus, seeds are rendered light-hard upon exposure to light for a minimum of approximately 32 hours.

It is clear that during darkness certain processes take place which lead ultimately to germination and that these processes are nullified by light. An

TABLE II

The effect of a 'light-break'

Treatment (cycle)		Percentage germination
Light period (hrs.)	Dark period (hrs.)	
6	18	85·3
7	17	86·4
6	18 (with 1 hr. light-break)	30·3
20	4	25·0
Continuous	—	27·7
—	Continuous	89·0

investigation was made of the minimum period of darkness required by the seeds, from the time of sowing, for germination to occur, and it was found that maintenance in darkness for 32 hours permits the germination of most of the viable seeds. After this time has elapsed light has little or no inhibitory effect and it seems likely therefore that the germination process is then irreversible.

The effect of various spectral regions

To determine the wavelength components of white light that are responsible for the inhibitory effects described above, a series of experiments was carried out using monochromatic light. Such light was obtained from a 1,000-watt tungsten-filament bulb used in conjunction with a series of Schott line interference filters having a band-pass of 10–14 mμ at half-maximum transmission. The intensity of irradiation, which was measured by means of a photo-cell first calibrated against a thermopile for each interference filter, was regulated by varying the distance of the seeds from the source. The filters were protected from excessive heat by interposing a square of Chance heat-absorbing glass (O.M. 20) between the lamp and each filter. The heat-absorbing glass and filters were cooled by an air-stream from a high-velocity fan. The whole equipment was housed in a room thermostatically controlled at 22° C.

In the present experiment one dish of seeds was placed under each filter, the intensity of irradiation being adjusted to 800 ergs./cm.^{2} sec. at seed level. The seeds were exposed to the various spectral regions for 72 hours, after which time a high percentage of those seeds maintained in darkness was

found to have germinated. From the results shown in Table III it is seen that there is marked inhibition of germination at 452 mμ and 710 mμ, and less inhibition in the blue-green region (483–96 mμ); the inhibition in the green and red wavelengths (542–651 mμ) is only slight.

Effect of duration of irradiation with blue and infra-red regions

The above experiment shows that blue and infra-red radiation (452 mμ and 710 mμ respectively) markedly inhibit germination. The present experiment was carried out to ascertain whether photoperiodic effects are given by these spectral regions alone.

TABLE III

The inhibitory effect of various spectral regions

Wavelength (mμ)	Percentage germination
452	16·7
483	59·5
496	49·1
542	74·7
577	74·8
596	88·0
651	60·8
710	12·2
Dark control	89·6

TABLE IV

Effect of daily irradiation with blue light

Daily light period (hrs.)	Percentage germination
8	46·2
12	25·1
16	11·2
20	1·4

In this experiment, blue light was obtained from blue fluorescent tubes in conjunction with blue perspex and a sheet of proprietary blue celluloid, this combination transmitting a negligible amount only of infra-red radiation. The intensity at seed level was 1,600 erg./cm.^{2}/sec. Seeds were exposed to three cycles composed of daily photoperiods of various durations. Distinct photoperiodic effects, comparable with those obtained using 'daylight' fluorescent tubes, are seen clearly from Table IV. When the effect of single periods of blue light was tested it was found that at least 30 hours of irradiation were required in order to obtain more than 50 per cent. inhibition of germination. These results also agree well with those obtained with white fluorescent light.

Similar experiments to those described for blue light were carried out using infra-red radiation. The latter was obtained by using a combination of red (R400) and blue (B700) perspex with a 100-watt tungsten-filament lamp and a 9-cm. water screen. This combination transmits a rather wide band of radiation from 650 mμ to 1,000 mμ, thus covering the active region of the infra-red (710 mμ). The seeds were maintained at a distance of 40 cm.

from the lamp but the intensity at this level was not known. In the first experiments it was found that when seeds were exposed to various daily periods of irradiation (the treatment being repeated for 3 cycles) there was almost complete inhibition of germination at all the periods tested. In the second experiment, when periods of daily irradiation shorter than 4 hours were used, appreciable inhibition was obtained even with 30 minutes of infra-red radiation each day. Single periods of irradiation with infra-red also were found to be more effective than blue light. Exposure of the seeds to infra-red for 12 hours from sowing resulted in the same order of inhibition as that produced by 30 hours of blue light, and almost complete inhibition is given by a single 17-hour period of infra-red radiation.

TABLE V

The effect of temperature on the germination of light-hard and thermodormant seeds

Temperature	Percentage germination	
	Light-hard	Thermodormant
21° C.	0	0
18° C.	30·4	10·3
15° C.	73·2	29·6
10° C.	89·4	82·6
3–5° C.	92·4	95·3

The effect of temperature treatment on light-hard and thermodormant seeds

It has been shown above that when seeds whose germination has been inhibited by light at 21° C. are transferred into darkness at the same temperature they do not germinate; these seeds are described as being light-hard. Similarly, it has been found that seeds inhibited by high temperatures in darkness do not germinate even when the temperature is afterwards lowered to 21° C. When temperatures lower than the critical region of 21° C. were tested, it was found that germination of light-hard and 'thermodormant' seeds then takes place. The results presented in Table V show this for seeds that were rendered light-hard by exposure to white fluorescent light for 3 days at 21° C. Thermodormancy was induced by holding seeds in darkness for three days at 28°–30° C.

DISCUSSION

From an empirical viewpoint it seems clear that the responses of these seeds to various light régimes, using 'daylight' fluorescent light, represent a form of photoperiodic behaviour. Indeed, many of the characteristic features of photoperiodism, originally demonstrated with the flowering response of herbaceous plants, can be seen in the phenomenon under study here. For example, the absolute length of the dark period would appear to be of prime importance in controlling germination, and, with certain qualifications, variation in the duration of the photoperiod has little effect.

However, certain facts emerge from this investigation which make it doubt-

ful whether we are dealing with photoperiodism in the generally understood sense of the term. In examining the effects of different spectral regions it has been found that both blue and infra-red radiations markedly inhibit germination when continuous irradiation or rather long durations are employed. It has also been shown that the effect of infra-red is much stronger than that of blue light. (Our failure to obtain inhibition with infra-red in previous experiments (Black and Wareing, 1957) can probably be attributed to the use of radiation composed largely of wavelengths longer than 710 mμ.) Now since the infra-red content of white fluorescent light is very small (e.g. Stolwijk, 1954) the inhibitory effect of such light can be ascribed to the blue region of the spectrum. In fact, a close parallelism exists between the photoperiodic effects obtained with white fluorescent and blue light (p. 35). However, no such correspondence was noted when infra-red radiation was tested, there being marked inhibitory effects even with very short daily exposures. Presumably, if white light containing a relatively large proportion of infra-red radiation were used (e.g. direct sunlight) the responses of the seeds should be quite different from those described above. Thus, the photoperiodic behaviour of this material is dependent upon exposure to light of a rather limited spectral composition.

It is of interest to note here that with regard to the effective wavelengths the situation in *Nemophila* is somewhat similar to that described by several Dutch workers for the photoperiodic control of flowering in certain species (see e.g. Wassink and Stolwijk, 1956). The analogy does not extend far, however, for in *Nemophila* there is no photoperiodic effect using infra-red radiation. It should also be mentioned that in recent studies with reliable light sources, it was found that inhibition of *Lamium* seeds is brought about only in infra-red radiation (Baxter-Jones and Bailey, 1956). The marked physiological role of blue light in *Nemophila* is therefore of considerable interest.

Our experimental results do not allow of an interpretation in simple terms of the photomorphogenic pigment system proposed by Borthwick *et al.* (1954) to participate in the germination of light-requiring seeds and in photoperiodic phenomena. However, it has been pointed out by Hendricks (personal communication) that the action spectrum for the inhibition of seed germination and root elongation in *Nemophila* closely resembles that for anthocyanin synthesis (Siegelman and Hendricks, 1957). It has been suggested that these effects are due to varying degrees of simultaneous excitation by high irradiances of both the red and infra-red absorbing forms of the pigment and are related to the ratio between the concentration of substances that react with the two pigments. This would explain why we have found the maximum inhibitory effect in *Nemophila* to be at 710 mμ, and not 730 mμ as would be expected if there were absorbtion only by the infra-red absorbing form (Black and Wareing, 1957). The effectiveness of blue cannot yet be fully accounted for.

Since high temperatures prevent germination even in darkness it would

appear that a temperature-dependent inhibitory reaction occurs. Whether or not this is directly associated with the pigment is not clear. It has been shown that removal to temperatures lower than 21° C. in darkness is the only requisite for the germination of 'thermodormant' seeds. An explanation which would be consistent with this finding is that competitive reactions having different temperature-coefficients are differentially affected by temperature changes. Thus, it may be that the course of the germination reactions is altered by high temperatures so that the normal end products are never attained. However, at low temperatures the germination reactions are favoured.

Since the curves of germination in light and darkness are more or less parallel (Fig. 1) it appears that light enhances the effect of increasing temperature. At 21° C. a certain threshold in the combined effects is reached which prevents germination.

The experimental results show that the seeds exhibit what appears to be true photoperiodic behaviour in white fluorescent and blue light. A characteristic feature of control by the daylength—the importance of the absolute length of the day period—has been found for the germination responses of these seeds. In short dark periods germination is inhibited even though the associated photoperiods are also short. Further, moderately long photoperiods (of, say, 20 hours' duration) fail to inhibit appreciably germination when the accompanying dark period is long. As in the flowering response of herbaceous plants, the effect of the dark period is nullified by a 'light-break'. However, although the duration of the dark period is of prime importance there is also a marked effect of excessively long photoperiods, even when combined with long dark periods (p. 31). This would appear to be analogous with the falling phase of the photoperiod seen in some plants, e.g. Biloxi soybean (Hamner, 1940) and with the effect of long light periods on germination of birch (Black and Wareing, loc. cit.).

A full interpretation, accounting for all the above results in terms of the reversible pigment system, is not possible with the available data. However, one plausible assumption is that the dark period is a measure of the time available for the promotive form of the pigment to expedite germination, either directly or indirectly. Irradiation with blue or infra-red causes the reversion of this pigment to the non-promotive form, infra-red being more effective in this respect (Wareing and Black, 1958).

It is perhaps unprofitable to speculate at length on the possible explanations. This will have to await a fuller analysis of the partial processes taking place in these seeds.

SUMMARY

In seed of *Nemophila insignis*, germination takes place in short days, but, above a certain critical temperature, is prevented by long daily photoperiods. As the temperature increases, germination even in darkness is suppressed. These are true photoperiodic effects and the germination of a given population is determined primarily by the absolute length of the dark period. However,

the photoperiodic responses depend on the spectral composition of the light and are obtained only with white light of low infra-red content (white fluorescent) or with blue 'monochromatic' light. Infra-red radiation (ca. 710 mμ) is strongly inhibitory, single exposures of relatively short duration almost completely suppressing germination.

LITERATURE CITED

BAXTER-JONES, M., and BAILEY, L. F. (1956). Light effects on the germination of seeds of Henbit (*Lamium amplexicaule* L.). *Plant Physiol.* **31**, 347.

BLACK, M., and WAREING, P. F. (1954). Photoperiodic control of germination in seed of birch (*Betula pubescens* Ehrh.). *Nature*, **174**, 705.

—— —— (1955). Growth studies in woody species. VII. Photoperiodic control of germination in *Betula pubescens* Ehrh. *Physiol. Plantarum*, **8**, 300.

—— —— (1957). Sensitivity of light-inhibited seeds to certain spectral regions. *Nature*, **180**, 395.

BORTHWICK, H. A., HENDRICKS, S. B., PARKER, M. W., TOOLE, E. H., and TOOLE, V. K. (1952). A reversible photoreaction controlling seed germination. *Proc. Nat. Acad. Sci.* **38**, 662.

—— —— TOOLE, E. H., and TOOLE, V. K. (1954). Action of light on lettuce-seed germination. *Bot. Gaz.* **115**, 205.

HAMNER, K. C. (1940). Interrelation of light and darkness in photoperiodic induction. Ibid. **101**, 658.

ISIKAWA, S. (1954). Light sensitivity against germination. I. 'Photoperiodism' of seeds. *Bot. Mag. Tokyo*, **67**, 51.

LEHMANN, E. (1909). Zur Keimungsphysiologie und -biologie von *Ranunculus sceleratus* L. und einiger anderer Samen. *Ber. d. d. bot. Ges.* **27**, 476.

SIEGELMAN, H. W., and HENDRICKS, S. B. (1957). Photocontrol of anthocyanin formation in turnip and red cabbage seedlings. *Plant Physiol.* **32**, 393.

STOLWIJK, J. A. J. (1954). *Wavelength dependence of photomorphogenesis in plants*. Wageningen.

WAREING, P. F. (1953). Growth studies in woody species. V. Photoperiodism in dormant buds of *Fagus sylvatica* L. *Physiol. Plantarum*, **6**, 692.

—— and BLACK, M. (1958). Similar effects of blue and infra-red radiation on light-sensitive seeds. *Nature*, **181**, 1420.

WASSINK, E. C., and STOLWIJK, J. A. J. (1956). Effects of light quality on plant growth. *Ann. Rev. Plant Phys.* **7**, 373.

The Spectral Sensitivity of Light-induced Leaf Movements

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SUMMARY

White, blue, green, red, and deep red illumination were used to shorten a dark period for plants of *Bauhinia monandra* under 12 hour:12 hour alternations of white light and darkness. Red alone imitated the effect of white light in advancing the cycle of leaf movement. Deep red did not counteract the effect of previous illumination with white light.

INTRODUCTION

WITH the exception of tropisms alone, all growth responses to illumination appear to have essentially identical action spectra. Thus seed germination, the light-growth effect, and photoperiodism have all been reported to be controlled by relatively small intensities of red light but to be insensitive to wavelengths less than about 520 mμ. In each case the effect of red light can be counteracted by exposure immediately afterwards to deep red (of which 730 mμ is the most active wavelength) (Flint and McAllister, 1935). It has been concluded (Borthwick *et al.*, 1950) that the same pigment system is responsible for every one of these responses.

Although leaf movements are not always caused by growth, they too have been reported to have this same spectral sensitivity. Bünning and Lörcher (1957) have shown that after plants of *Phaseolus multiflorus* have been kept in the dark until leaf movement stops, it can be started again by white or red (610–690 mμ) light but not by green or blue. Once again, although deep red light could not make the leaves begin moving, it could, on the other hand, prevent the response to red. His results 'imply that in the regulation of endogenous diurnal rhythms . . . the same red-deep red pigment system is at work as . . . in other physiological light reactions'. (My translation.)

However, there is one other published account of the spectral sensitivity of leaf movements, that of Fortanier (1954) on *Arachis*, in which movement was reported to be brought about by blue light and not by red. I have now tested the sensitivity of *Bauhinia monandra* Kurz, a plant used before (Holdsworth, 1956, 1959*a* and *b*) to test relationships between photoperiodism and leaf movements.

EXPERIMENTAL

The method Bünning and Lörcher used was not copied exactly. Instead the treatments correspond with those used by Funke (1936) and Withrow and

Benedict (1936) to test the colour sensitivity of photoperiodism, in which coloured light was used only to extend the daylength.

The plants were illuminated naturally (i.e. about 12 hours daily) until the first leaf had been expanded for a week. Then one night was shortened by illumination with either white, blue, green, red, or deep red light from midnight; illumination was continued with white light after 6 a.m. and the leaf position photographed at 10-minute intervals when the leaves next began to close. It was known (Holdsworth, 1959*a*) that following illumination at 6 a.m. the leaves are nearly half closed by 6 p.m. and that advancing the time of illumination to midnight with *white* light, advances the closing time by about $4\frac{1}{2}$ hours.

Four plants were used for each treatment and the treatments were tested in pairs in all possible combinations.

No attempt was made to equalize the energies of the different colours exactly. No equipment was in any case available with which to do so; and fortunately it was also known that even very weak white light applied at midnight will make the leaves close early.

The red and deep red illumination were produced by a Kodak 1 safelight and an 88A Wratten filter respectively over filament lamps screened also with 2 cm. of water. The blue and green illumination came from Wratten filters 47B and 45 respectively over appropriate coloured fluorescent tubes. According to the manufacturer's published data (Kodak, 1953, 1958) No. 45 transmits from 440 to 540 mμ, 47B from 360 to 500, and 88A from 725 upwards. The safelight (personal communication) transmits from 600 mμ upwards.

The effect of deep red after white light was also tested, but again not in the same way as by Bünning and Lörcher. They exposed their plants for 12 hours to red before changing to deep red. After 12 hours in red (or white) light *Bauhinia* leaves would already be shutting and my type of test would be inapplicable. Instead, as it was known (Holdsworth, 1959*a*) that appreciable earlier closing can be induced by a light break of 4 hours in the previous night, an effect of deep red continued until 6 a.m. after a 4-hour break (10 p.m. to 2 a.m.) was looked for.

In the discussion will be found mention of observations of leaf movements in continuous 'darkness'. These were obtained with the automatically triggered camera mentioned in my paper of 1959(*a*). A synchronized flash of weak green light was used to make the exposures.

RESULTS

Fig. 1 shows quite clearly that of the four colours used, only red has the same effect as white light in making the leaves close early the following afternoon. It must be mentioned that the green was rather bluer than that used by Bünning and Lörcher. Illumination from a naphthol green B safelight (Withrow and Price, 1957) which has nearly the same transmission as the filter Bünning and Lörcher used (450–610 mμ) was, as a matter of fact, found to advance the time of closing appreciably, though admittedly not so much

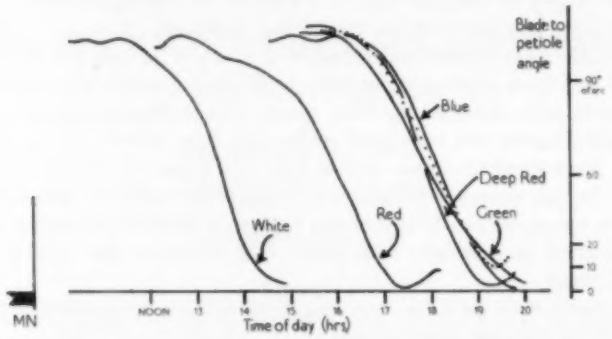

FIG. 1. Closing of *Bauhinia monandra* leaves after illumination with different colours.
After putting in the dark at 18.00 hours, the plants were illuminated with coloured or white light from midnight to 6.00 hours, after which all were illuminated with white light for the rest of the day. Records were made as the leaves shut the following afternoon. For comparison, the dotted line shows the closing of leaves illuminated only from 6.00 hours. (The difference between the times of closure of 'Red' and 'White' is explained by a considerable difference in intensity of white from the rest.)

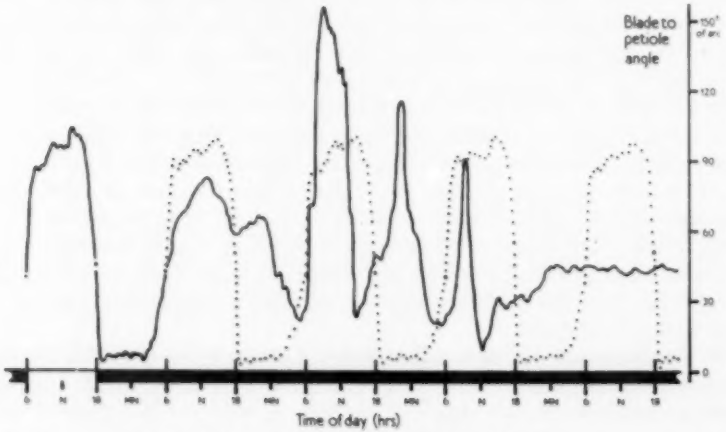

FIG. 2. Movements of *Bauhinia monandra* leaves in continued darkness.
For comparison, the movements under 12-hour alternations of light and darkness are shown dotted. (Previously all the plants had experienced similar alternations.)

as the red. It was discovered that Withrow and Price's safelight has, however, some light-growth effects and causes some stimulation of extension growth in maize coleoptile sections.

If, during the night, plants are exposed for 4 hours to white light the leaves close about 1½ hours early the next evening (Holdsworth, 1959*a*). Exposing them for the following 4 hours to deep red light did not prevent this.

DISCUSSION

The diurnal movements of the leaves of *Bauhinia monandra* are seen to be controlled by the red component of white light and not by shorter wavelengths. In this they agree with those of *Phaseolus* reported by Bünning and Lörcher (1957). In *Arachis hypogaea*, on the other hand, Fortanier (1954) found blue and not red caused movement. There are exceptions to the rule that blue light is ineffective in photoperiodism but not among Leguminosae (Funke, 1936; Wassink *et al.*, 1950). A close reading of Fortanier's paper suggests that he may have observed photonastic movement and not photoregulation of diurnal movements. That photonasty may have a different spectral sensitivity from diurnal movements is not improbable, and indeed Bünning and Lörcher record that not all aspects of the control even of diurnal movements have exactly the same responses. The colour sensitivity of nastic movement would not be adequately assessed without close control of the intensity of each colour and that was not possible in my tests. It was, however, noticed that after exposure to every one of the colours used, including deep red, the leaves were appreciably more open by 6 a.m. than those kept throughout the night in darkness.

Although the diurnal movements of *Bauhinia* are like those of *Phaseolus* in being sensitive to red light, complementary effects of deep and bright red could not be demonstrated. In photoperiodism, demonstration of the antagonism between red and deep red is confined to their action in the light-break effect (Downs, 1956). According to Bünning (1948, p. 377) there is also a light-break effect on diurnal leaf movements with as little as 1 minute illumination. On *Bauhinia*, however, I was not able (1959*a*) to confirm this with interruptions of the dark period which are effective in photoperiodism. Indeed the 'break' has to be a quarter or even a third of the whole dark period before a clear effect becomes apparent. It has been shown here, that deep red could not prevent the effects of such long breaks as this, when the exposure to deep red followed the break. Bünning and Lörcher found in *Phaseolus* that deep red counteracted the effect of red not only when given subsequently, but also when plants were exposed to both at the same time. This cannot be true of *Bauhinia*, for both the white and red sources must have supplied abundant radiation in the 700 mμ band. The water screens would only have removed long wave infra-red.

Bünning and Lörcher's demonstration of the annulment of red light effect by deep red was made after even longer (12 hours) exposure to red light than in my experiments; but, on the other hand, the ensuing leaf movements were recorded in darkness. I have shown (1959*b*) that in *Bauhinia*, movements continuing in darkness are somewhat different from those that occur in alternations of darkness and light. It seemed worth while testing whether shorter light breaks would interfere with the cycle of movement in continuous darkness, and if so, whether deep red would counteract them.

In the experiments reported in 1959(*b*) movements in darkness were

followed only for 24 hours. Now, with the knowledge that green is a safe light for the observation of leaf movements, it is possible to keep records easily over longer periods. The record shown in Fig. 2 was obtained, therefore, by photography in green light. It can be seen that only during the first 24 hours is there any suggestion of a 24-hour cycle. Subsequently, violent movements up and down at more frequent but irregular intervals set in. These, in turn, continue only a day, or sometimes 2 days longer, before movement ceases altogether. Light breaks (an hour at midnight), even though repeated each night, did not appear to have any effect on the course of events—although admittedly, comparison was difficult for, as the dark period continued, individual leaves no longer moved in step. There was, in any case, no point in looking for an antagonistic effect of deep red light.

Diurnal rhythm is obviously weak in *Bauhinia*, if it exists at all. It appears that *Bauhinia* very rapidly passes into a state of 'rigor', for it was only during the first night in darkness that there was even a nastic response to the light break.

LITERATURE CITED

BÜNNING, E. (1948). *Entwicklungs- und Bewegungsphysiologie der Pflanze*. Berlin.

—— and LÖRCHER, L. (1957). Regulierung und Auslosung endogen-tagesperiodischer Blattbewegungen durch verschiedene Lichtqualitaten, *Naturwiss.* **17**, 1–2.

BORTHWICK, H. A., PARKER, M. W., HENDRICKS, S. B. (1950). Recent developments in the control of flowering by photoperiod, *Amer. Nat.* **84**, 117–33.

DOWNS, R. J. (1956). Photoreversibility of flower initiation, *Plant Phys.* **31**, 279–83.

FLINT, L. H., MCALLISTER, E. D. (1935). Wavelengths of radiation in the visible spectrum inhibiting the germination of light-sensitive lettuce seed, *Smiths. Inst. Pub. Misc. Coll.* **94** (5).

FORTANIER, E. J. (1954). Some observations on the influence of spectral regions of light on stem elongation, flower bud elongation, flower bud opening and leaf movement in *Arachis hypogaea* L., *Mededel. v.d. Landbouw. t. Wageningen, Ned.* **54**, 103–14.

FUNKE, G. L. (1936). Proeven over photoperiodiciteit by verschillend gekleurd licht, *Biol. Jaarb. Nat. Genoot. Dodonaea*, **3**, 225–61.

HOLDSWORTH, M. (1956). Leaf movements and the *Tagesrhythmik* explanation of photoperiodism, *Nature*, **177**, 845–6.

—— (1959*a*). The effects of daylength on the movements of pulvinate leaves, *New Phytol.* **58**, 29–45.

—— (1959*b*). The effect of length of dark period on the amplitude of leaf movement in *Bauhinia monandra*, *J. Exp. Bot.* **10**, 104–8.

KODAK LTD. (1953). *Wratten light filters*. London.

—— (1958). *Kodak data sheets*. London.

WASSINK, E. C., SLUYSMAN, C. M. J., STOLWIJK, J. A. J. (1950). On some photoperiodic and formative effects of coloured light in *Brassica rapa f. oleifera, subf. annua*, *Proc. Ned. Akad. Wetens. Amsterdam*, **53**, 1466–75.

WITHROW, R. B., BENEDICT, H. M. (1936). Photoperiodic responses of certain greenhouse annuals as influenced by intensity and wavelength of artificial light used to lengthen the daylight period, *Plant Phys.* **11**, 225–49.

—— PRICE, L. (1957). A darkroom safelight for research in plant physiology, *Plant Phys.* **32**, 244–8.

Morphogenetic Studies on *Osmunda cinnamomea* L.

THE AUXIN RELATIONSHIPS OF EXPANDING FRONDS

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SUMMARY

Net production of diffusible auxin by fronds of *O. cinnamomea* has been found to extend throughout the period of final expansion, with a maximum which occurs shortly after the rate of elongation has reached its maximum and begun to decline. Auxin is apparently produced only in the pinnae, and from these it enters the rachis where its movement is polar. Diffusible auxin moves through the rachis without significant loss except in the region of extensive elongation where a certain amount disappears. No such disappearance was found in ether extraction studies. Moreover, in certain cases, ether-extractable auxin persists for as much as 48 hours in the rachis after removal of the auxin source, although none can be obtained by diffusion under these conditions. It is concluded that diffusible auxin is more closely related to growth phenomena in the frond than is extractable auxin. In many cases, there is an apparent failure of transport in the leaf base which reduces or eliminates expected auxin yields by diffusion at this level. Diffusible auxin is an essential participant in three morphogenetic phenomena in the developing frond: rachis elongation, crozier uncoiling, and final differentiation of xylem and hypodermal sclerenchyma. In none of these can it be considered as a specific determining factor since the response to auxin depends upon the physiological state of the reacting cells. In the intact frond diffusible auxin is present in excess of the minimum required for both rachis elongation and uncoiling. Several aspects of the relationship between auxin distribution and development in the frond are discussed.

INTRODUCTION

THE vegetative leaves of the fern *Osmunda cinnamomea* L. undergo an extensive development involving long continued apical growth, active cell division in the leaf axis, and ultimately rapid and extensive cell elongation (Steeves and Briggs, 1958 ; Briggs and Steeves, 1958). During the first three growing seasons in the life history of an individual vegetative frond, and the early part of the fourth, development proceeds at an extremely slow rate. In the latter part of the fourth season, apical growth is accelerated and a crozier with lateral leaflets or pinnae is produced. During the fifth and final growing

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season there is a truly dramatic enlargement in which the frond uncoils, expands, and ultimately matures (Briggs and Steeves, 1958, 1959). On the other hand, the stem or rhizome is characterized by an almost complete absence of internodal elongation so that the short shoot habit is the permanent pattern of this species (Steeves and Wetmore, 1953).

In seeking an interpretation of the growth habit of this plant and of the rather complex sequence of events involved in the development of its leaves, attention was naturally turned to the possible role of auxins, the best known growth-controlling substances in the higher plants. So little is known about the growth hormone relationships of the ferns, in contrast to the seed plants, that such a study should have general interest beyond the problems of a single species. The present paper will report the auxin relationships of vegetative fronds during final expansion and maturation in the fifth growing season.

Previous studies on the occurrence and activity of growth hormones in ferns, although scanty, suggest relationships which are in a general way similar to those found in the seed plants. Prankerd (1922) and Du Buy and Nuernbergk (1938) have provided evidence that the leaves of certain ferns display both phototropism and geotropism which, like similar phenomena in higher plants, are related presumably to the action of growth hormones. In the fern *Pteris longifolia*, Albaum (1938) was able to demonstrate, both by diffusion and by extraction, that the apical region of the prothallus produces auxin which is transported basipetally and has an inhibitory effect upon adventitious outgrowths in the basal region. Similarly, he extracted auxin from leaves of young sporophytes and showed that this auxin is responsible for an inhibition of growth of the prothallus, to which the sporophyte is attached, as well as of younger sporeling leaves. Wardlaw (1946) showed that, as in the higher plants, applied indoleacetic acid can replace the shoot apex in the inhibition of lateral bud development in certain ferns. Evidence for the role of growth hormones in the development of the fern *Marsilea drummondii* has been provided by Allsopp (1954, 1956) who observed a marked influence of indoleacetonitrile upon internodal elongation and leaf development of sporelings growing in sterile culture. From the point of view of the present study, the most pertinent investigation is that reported by Wetmore and Pratt (1949), for *Adiantum pedatum*. These authors showed that leaves at all stages of development provide a supply of diffusible auxin to the rhizome beyond that which disappears during the development of the leaf itself. They concluded that auxin entering the rhizome from developing leaves is correlated with a general absence of buds in the apical region of the rhizome and with internodal elongation.

Three earlier reports pertaining to the present study (Steeves *et al.*, 1953; Briggs *et al.*, 1955*a* and *b*) have dealt with an auxin-inactivating enzyme system in *O. cinnamomea*. This system is considered to be operative only at cut surfaces and in tissue breis and is not thought to play any part in the normal growth of the plant. In the course of these earlier studies, it was shown that expanding fronds produce considerable quantities of auxin obtainable either by diffusion or by ether extraction. A brief survey of auxin distribution in

growing fronds and in the rhizome was presented. The present article, and a second which will follow it, will attempt to provide a more complete picture of auxin relationships in the development of this unusual plant.

MATERIALS AND METHODS

The plants used in this study were collected from natural stands near Boston, Massachusetts. For the most part they were gathered in the fall after the onset of dormancy, stored in a cold room at approximately 4° C., and grown in a greenhouse in the spring. Some plants were brought directly to the greenhouse from the field in the spring before the breaking of dormancy. Auxin assays were carried out from late February to late June; and in the early part of the season supplementary illumination was provided.

In auxin diffusion studies, fronds were collected just prior to use and whole fronds or parts were placed singly or in groups with their cut basal ends on large ($8 \times 11 \times 1{\cdot}5$ mm.) blocks of 1·5 per cent. agar. Diffusions were carried out in moist chambers in the laboratory, protected from strong illumination. Following the diffusion, the blocks were assayed by the standard *Avena* test (Went and Thimann, 1937). Auxin yields have been expressed as average curvatures of the 12 *Avena* plants used in each assay. In each experiment a sensitivity block containing 0·025 mg./l. indoleacetic acid (IAA) was included and, where comparisons are made between experiments, curvatures have been corrected for variations in sensitivity.

The occurrence of an auxin-inactivating enzyme system at cut surfaces made it necessary to use the potassium cyanide technique previously described (Steeves *et al.*, 1953). It had been shown that the potassium cyanide has no harmful effects upon the diffusion, and, following the addition of ferrous sulphate to the agar blocks, no deleterious activity in the *Avena* test. Auxin extractions were carried out at 0° C. for 4 hours with freshly distilled, peroxide-free diethyl ether according to methods described earlier (Briggs *et al.*, 1955*b*). Where an external source of auxin was required, this was provided by the application of a paste containing 0·5 per cent. IAA by weight in a lanolin and water emulsion. Microtome sections for histological study were prepared by standard methods, as described in a previous paper (Steeves and Briggs, 1958).

AUXIN RELATIONSHIPS OF EXPANDING FRONDS

The net production of diffusible auxin. The initial step in the analysis of auxin production and distribution in expanding fronds during the fifth growing season was a determination of net production of diffusible auxin; that is, diffusible auxin beyond that which may be removed from the transport system in the rachis at various stages of development. Because of differences in size and rate of development between plants, it would have been desirable to use a sequence of fronds from a single plant in measuring auxin production at successive stages. Ordinarily, however, the number of vegetative fronds produced by a single plant is not sufficient to permit a reasonable test to be

made. Consequently, at the outset of the experiment, the dormant plants were grouped in pairs which were matched for size as far as possible. In the main experiment devoted to this analysis, 5 replicates, that is, 5 pairs of plants, were used. The first assay was made 3 days after the plants had been forced, and diffusions were carried out at intervals of 3 to 7 days thereafter for a period

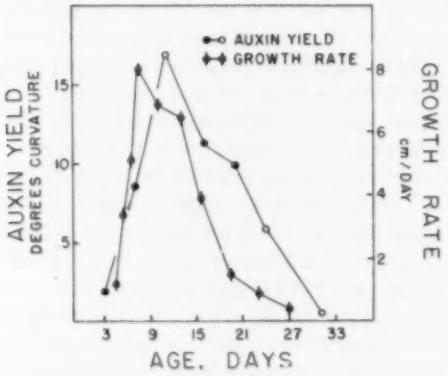


FIG. 1. Diffusible auxin production and growth-rate of vegetative fronds during their fifth growing season. Data from one matched pair of plants. Growth-rate measurements from a single frond. Solid and open circles indicate from which of the two plants of the matched pair the individual frond used for diffusion was obtained.

of 28 additional days. On each occasion one frond was taken from each pair and was diffused separately on a large agar block at ground level, that is, just above the morphologically distinct leaf base. From the point of view of determining the amount of auxin which actually enters the rhizome it would have been preferable to diffuse from the actual point of attachment to the rhizome; but such a procedure would have made it necessary to dissect the plant completely so that successive fronds from the same plant could not have been used. One frond in each pair of plants was selected for growth measurements, and daily records of its height were kept. The entire experiment was repeated on two separate occasions, with a total of 8 replicates.

In spite of fluctuations resulting primarily from the nature of the material, a definite trend in net auxin production appears. In Fig. 1 the auxin production by fronds of one of the replicate pairs is shown in the form of a graph, together with the growth rate of one of the fronds of that pair. These results are representative of those from the 8 replicates. It may be seen that measurable quantities of auxin are produced by the frond for a period of approximately a month, during which the frond is actually growing. The production begins at a relatively low level, rises to a peak at about the time that half of the pinnae have been set off by the uncoiling crozier, or a little later in some

cases, and then declines gradually during the remainder of uncoiling and expansion.

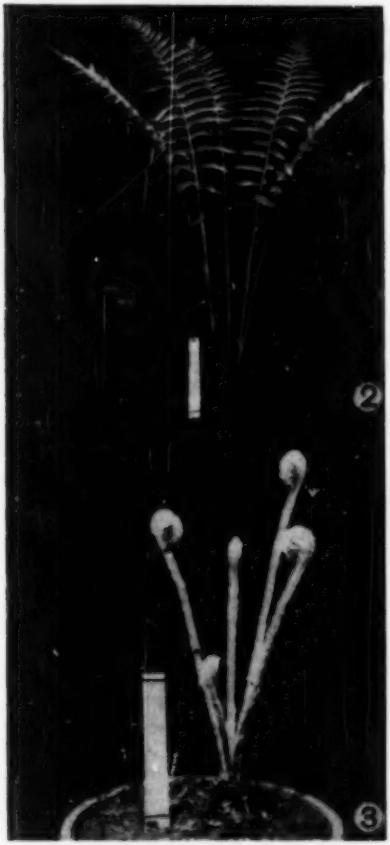
A comparison between the curves for auxin production and growth-rate reveals an interesting relationship between the two phenomena. The low initial production of auxin is correlated with a slow rate of development. Both auxin production and growth rate then rise sharply to their respective maxima; but growth-rate increases more abruptly, and has reached its peak and begun to decline before maximum auxin production is attained. The decline in growth-rate is distinctly in advance of the decline in auxin production, but both phenomena have, as might be expected, essentially the same overall duration. This same relationship has been found in all 8 replicates. The values recorded in this experiment do not necessarily represent the total auxin production by the fronds, since any auxin removed from the transport system in the growing frond would not reach the level at which diffusions were carried out. It is, therefore, possible that the peak of net auxin production does not correspond to the maximum of total production. Conceivably, net auxin production may reach a peak only after growth has diminished.

In *O. cinnamomea*, spores are produced on separate fertile fronds, or sporophylls, which are morphologically quite distinct, are somewhat shorter than vegetative fronds, and wither after shedding spores in late spring. As part of the analysis just described, net auxin production by fertile fronds was determined for the same groups of plants. The production of auxin follows essentially the same pattern as that shown for vegetative fronds; but the maximum yield is only a little more than half that obtained in the previous case. Also, after the maximum is attained, auxin yield rapidly drops to zero as the spores mature and are shed.

The localization of auxin production in the expanding frond. In a study of auxin production and distribution in the leaf of tobacco, Avery (1935) concluded that the developing laminar tissue is the main source of the hormone. If a similar pattern were to be found in the leaf of *O. cinnamomea* during the final growing season, auxin production would be localized in the pinnae which contain all of the laminar tissue (Fig. 2). Growth at the apex is, at this time, concerned with the formation of a terminal pinna which need not be considered as different from the lateral pinnae. On the other hand, it has been shown that there is considerable cell division within certain portions of the rachis during final expansion (Briggs and Steeves, 1958) and the possibility of auxin production by this tissue must be considered.

In the final expansion of the *O. cinnamomea* frond, there is an initial phase during which elongation in the leaf axis elevates the crozier without uncoiling. This is followed by a phase of active uncoiling (Briggs and Steeves, 1958, 1959). Before uncoiling begins the pinnae are all contained within the crozier and the most basal pair is situated at least 180° (one-half coil) into the crozier from its base. As uncoiling progresses, the pinnae move successively to the outside of the crozier and ultimately are set off below it where they complete

their expansion. Fig. 3 illustrates fronds early in the uncoiling phase, on which no pinnae have yet been set off below the crozier. In initial experiments it was



FIGS. 2 and 3. FIG. 2. Plant of *O. cinnamomea* with fully expanded vegetative fronds. FIG. 3. Plant of *O. cinnamomea* showing developing fronds on which no pinnae have yet been set off below crozier. Marks on frond at left delimit approximately the zone of elongation. In Figs. 2 and 3 distance between black lines on label is 10 cm.

consistently shown that if, in comparable fronds in which no pinnae have yet been set off, auxin diffusions are made at the base of the crozier and at ground level, the yield at the crozier base is distinctly higher. This suggests that the auxin source is in the crozier at this time and that, rather than contributing additional auxin, the rachis below the crozier in some way reduces the supply.

In order to determine which part of the crozier actually produces the auxin, various numbers of pinnae were removed from croziers 18 to 21 hours prior to diffusion and the auxin yields were compared with those of intact controls. It is clear from the evidence presented in Table I that complete removal of all pinnae, including the terminal, reduces the auxin yield essentially to zero. On the other hand, if some pinnae are removed while others are left in place, a partial reduction is observed. At a later stage of development, after pinnae have been set off below the crozier, the excision of several pairs from the rachis outside the crozier likewise produces a distinct drop in auxin yield. It might be argued that traumatic effects or desiccation are responsible for the reduction in auxin yields in these cases. The likelihood of such effects was, however, reduced by the application of a lanolin paste to the cut surfaces following excision. Furthermore, it will be shown later in this paper that the rachis, even after complete removal of the pinnae, remains alive for several days and retains its ability to elongate in response to applied auxin.

TABLE I

Effect of removal of pinnae upon auxin yield

Stage of development	No. pinna pairs removed	Degrees curvature	Degrees curvature, intact control
No pinnae[a] set off	All + tip	0	—
No pinnae[a] set off	8	19·7	24·6
	12	8·1	24·6
4–7 pairs[b] pinnae set off	4–7	14·5	23·5

[a] 2 fronds.
[b] 3 fronds.

The evidence thus points to the pinnae as the major source of diffusible auxin in the expanding frond, although the possibility of a relatively minor production in the rachis within the crozier has not been eliminated. Experiments carried out for another purpose have shown that at least the outer 180° segment of the crozier may be removed without significantly reducing the auxin yield of the crozier (see Table IV), if the segment removed does not bear any pinnae. If, however, croziers are used at a slightly later stage of development when the outermost half turn of rachis bears several pairs of pinnae, the removal of that section markedly reduces auxin yield just as does the removal of pinnae. It would seem, therefore, that it is removal of pinnae rather than removal of rachis which accounts for the reduction in auxin yield.

The results of pinna removal experiments do not make clear whether the pinnae actually produce auxin which subsequently enters the rachis, or whether they are the source of a precursor substance which passes into the rachis and is there converted to auxin. In order to settle this point, an attempt was made to obtain auxin by diffusion directly from pinnae. Pinnae were

grouped in bundles and diffused on agar blocks. In these experiments fronds were used which had a number of pinnae set off below the crozier, because of the ease of excising pinnae under these conditions. It is clear from Table II that pinnae in various stages prior to final maturation, even after being set off below the crozier, produce considerable quantities of diffusible auxin. Under normal conditions, this auxin, in an active form, presumably passes into the rachis of the frond. Thus, the major production of auxin is associated with the development of laminar tissue, a process involving extensive cell division, as revealed by direct observation, even after the pinnae have been set off below the crozier.

TABLE II

Auxin diffusions from pinnae

	No. in sequence from base	No. pinnae per block	Degrees curvature
Exp 1. 8 pairs of pinnae set off	1	16	2·2
	3	16	4·9
	5	16	4·1
	8	16	8·3
Exp. 2. Completely uncoiled	1	16	5·7
	5	16	17·5
	9	16	11·5
	13	16	10·0
	17	16	1·1

Polarity of auxin transport in the rachis. Before proceeding with a consideration of the fate of auxin in the rachis, it seemed advisable to determine if the movement of diffusible auxin in the rachis is strictly polar. In initial experiments, fronds were selected on which 10 to 12 pairs of pinnae had been set off below the crozier and were borne on a section of rachis which was still elongating. The elongating segments with their pinnae were removed from the fronds and agar blocks were placed at both ends to collect any diffusible auxin. After a 3-hour diffusion period, the blocks were assayed (Table III). It is evident that in each case auxin appeared in the block at the basal end, but never in that at the apical end.

It has been demonstrated previously (Briggs *et al.*, 1955*b*) that externally applied IAA moves basipetally in the rachis of *O. cinnamomea* and may be collected by diffusion at the basal end of a segment to which it has been applied. It remained only to demonstrate that externally applied IAA does not move in an acropetal direction. On separate fronds a 5 mm. wide ring of IAA in a lanolin and water paste was applied either to the centre of the elongating region or to the non-elongating region below it. Fronds were selected for this experiment in which no pinnae had yet been set off below the crozier (Fig. 3). After 12 hours, cuts were made 4 or 5 cm. above and below the centre of the ring and the resulting segments were removed and diffused from their apical and basal ends. As in the previous cases, no auxin was ever collected from the

apical ends but was regularly collected from the basal ends. The auxin collected from the basal end was presumably a mixture of applied and endogenous auxin since no provision had been made to remove the native auxin from the rachis prior to the experiment.

TABLE III

Polarity of auxin transport in the rachis

Auxin source	Region of rachis	Length of segment, cm.	Auxin yield, degrees curvature	
			Apical	Basal
12 pairs pinnae	Zone of elongation	11	0	17·4
10 pairs pinnae	Zone of elongation	11	0	17·3
Applied and residual	Zone of elongation	8	0	22·7
Applied and residual	Zone of maturation	10	0·3	24·9
Applied and residual	Zone of maturation	10	0	32·0

From these experiments it may be concluded that the transport of auxin in the rachis of the expanding frond is strictly polar, and this conclusion applies equally well to endogenous auxin and to externally applied IAA, in both elongating and maturing regions.

The distribution of diffusible auxin in the rachis. The fate of diffusible auxin in the rachis was investigated by carrying out diffusion experiments at several distinctive locations along the rachis. In most cases fronds were used on which no pinnae had been set off below the crozier, so that the auxin source was concentrated within the crozier.

It has been noted earlier (Briggs and Steeves, 1958) that there is a certain amount of elongation in any given segment of rachis while it is contained within the crozier. This elongation, which involves both cell division and cell enlargement, is associated with the enlargement of the crozier before and during uncoiling. It is relatively small in comparison with that which occurs in the rachis just after it has been set off below the crozier, and which involves cell enlargement almost exclusively. There is a well-defined zone just below the crozier in any growing frond in which this extensive elongation takes place. This region is referred to as the zone elongation (see marks on left frond, Fig. 3). Below the zone of elongation is a region in which elongation has ceased and in which the final phases of tissue maturation are occurring. This non-elongating zone is referred to as the zone of maturation. Any given segment of rachis, after being set off from the crozier, is first in the zone of elongation and then in the zone of maturation.

In the first series of experiments, diffusions were carried out on comparable fronds or groups of fronds at ground level, at the base of the zone of elongation, at the base of the crozier (that is, just above the zone of elongation), and

one-half turn into the crozier. The leaves, diffused at a point one-half turn into the crozier, were carefully selected so that the discarded portion did not bear any pinnae. The results of a number of such diffusions are shown in Table IV. It is apparent that the auxin yield at the base of the crozier is essentially equal to that obtained from a point 180° into the crozier. It is clear that no diffusible auxin disappears in the outer one-half turn of crozier where relatively little elongation is taking place. On the other hand, the auxin yield at the base of the crozier is clearly greater than the yield collected below the zone of maximum elongation. In the region of the rachis in which active elongation is taking place, there thus appears to be a significant disappearance of diffusible auxin. On the other hand, within the limits of the accuracy of the methods, the diffusible auxin which enters the zone of maturation passes through the leaf axis to ground level without loss.

TABLE IV

Distribution of auxin in the rachis as determined by diffusion

Point of rachis at which auxin was collected	Degrees curvature[a] Experiment						
	1	2	3	4	5	6	7
180° into crozier	23·9[b]	10·0	30·0	—	—	—	—
Base of crozier	25·6	13·3	30·4	15·6[b]	23·6[b]	31·2[b]	20·7
Base of zone of elongation	8·7	—	—	3·9	13·5	13·3	6·5
Ground level	—	6·5	—	3·1	—	19·3[b]	5·6

[a] Experiment 3, 4 fronds, all others, 2 fronds.
[b] Standard errors between ±2·5° and ±3·5°. All others ±2·5°.

Just below ground level, the leaf axis becomes a broad flattened leaf base which undergoes essentially no elongation during final expansion of the fronds (Briggs and Steeves, 1958). In view of the absence of internodal elongation in the rhizome, it seemed necessary to investigate the fate of auxin in the leaf base, and particularly to determine whether it passes through this region into the rhizome. A series of experiments was therefore carried out in which auxin yield was determined in comparable fronds or groups of fronds at ground level, at the extreme base (point of attachment to the rhizome), and at various levels between these two points. The results, some of which are shown in Table V, form a rather confusing picture. In somewhat fewer than half of the total number of experiments done, nearly comparable yields were obtained at ground level and at the extreme base. Frequently, however, the yield from the base was drastically reduced; and in some cases no auxin was obtained at all. In a few instances auxin could be obtained from a point approximately midway in the leaf basal region, but not from the extreme base. It must be concluded that diffusible auxin can, in some cases, pass through the morphologically distinct leaf base in a manner similar to that in which it passes through the aerial portions of the rachis. However, the active operation of an auxin

transport system does not appear to be as constant a feature as it is in the upper portion of the leaf rachis (see next section).

TABLE V

Distribution of auxin in the leaf base as determined by diffusion

Point of leaf base at which auxin was collected	Degrees curvature[a] Experiment							
	1	2	3	4	5	6	7	8
Ground level	20·5	10·4	21·5	13·5	10·2	15·1	19·3	13·6
Intermediate between ground level and rhizome	—	8·4	—	—	12·9	—	15·6	—
Attachment to rhizome	0·5	10·9	16·5	0·5	1·2	18·3	2·6	10·2

[a] Experiment 6, 4 fronds, all others, 2 fronds.

The distribution of ether-extractable auxin in the rachis. Since the diffusion method presumably yields information only about auxin which is actually moving in the transport system, an attempt was made to extend the picture of auxin distribution in the developing frond by a study of ether-extractable auxin. The results of this analysis, shown in Table VI, extend and confirm the observation made earlier in another study (Briggs *et al.*, 1955*b*) that the distinct drop in auxin content in the zone of elongation, which appears in diffusion studies, cannot be detected by ether extractions made on the basis of weight of tissue. On the other hand, since it may be demonstrated that the rachis in the zone of elongation has a density nearly twice that of the rachis in the zone of maturation, it is quite possible that it is not valid to deal with auxin concentration per unit weight of tissue extracted. In any event, diffusible auxin is more readily related to the growth pattern of the frond than is extractable auxin.

TABLE VI

Auxin obtained by short-term ether extraction from various regions of developing fronds

Region extracted	Degrees curvature per 5 gm. tissue[a, b] Experiment	
	1	2
Crozier plus pinnae	15·6	17·4
Zone of elongation	22·5	
Upper zone of maturation	22·3	26·2
Lower zone of maturation	22·5	26·6
Leaf base	23·3	31·2

[a] All values given were substantially below the maximum curvature for the day.
[b] Maximum standard error $\pm 1{\cdot}6°$.

The data presented in Table VI do, however, contribute to an understanding of the frequent failure to obtain expected auxin yields by diffusion from the leaf base (see Table V). In one of the two experiments shown in Table VI, the auxin content of the leaf base is distinctly higher than in the leaf rachis above. In the other experiment, no such increase in auxin content is noted. In a further experiment not shown in the table, the leaf base was subdivided prior to extraction; and it was found that an increased auxin content occurred only in the lower portion of the base. These observations could be interpreted as evidence for an accumulation of auxin in the leaf base in certain cases such as might be expected if the normal auxin transport failed.

Another series of experiments has led to a further note of caution with regard to auxin extraction data. It has been reported earlier (Briggs *et al.*, 1955*b*) that if all laminar tissue is removed from fronds 48 hours prior to extraction, no auxin can be obtained from the zone of maturation. However, more recent experiments have shown that this is not always the case, although the reason for differences in behaviour at different times is not clear. Comparable fronds were selected on which no pinnae had been set off below the crozier, and the croziers were excised so as to leave approximately 180° of curved rachis, devoid of pinnae, surmounting the remaining bare rachis. Comparable samples were cut and extracted immediately, and after 6, 24, and 48 hours. In each case, the 180° segment from the crozier was extracted separately, and the remaining rachis was cut into a series of 2 cm. pieces which were also separately analysed (Table VII). Clearly, even after 48 hours, there is a con-

TABLE VII

Distribution of ether-extractable auxin in rachis following excision of crozier. Details in text

Region extracted	Degrees curvature per 3 gm. tissue Time from removal of crozier, hours			
	0	6	24	48
Outer 180°	22·2	14·4	10·9	7·7
Remaining rachis segments:				
1	22·4	17·6	13·8	14·8
2	22·4	16·7	15·2	14·7
3	20·9	22·2	16·8	18·1
4	18·9	17·4	13·3	13·2
5 (Basal)	26·4	18·0	11·7	15·9

siderable amount of ether-extractable auxin present throughout the rachis, although the content is diminishing. Another similar experiment has shown a comparable persistence of extractable auxin in the leaf base after the removal of the source; but this evidence is complicated somewhat by the possibility of a failure of auxin transport in the leaf base which has previously been mentioned. It would thus appear that under certain circumstances, not as yet

understood, auxin may persist in the rachis long after the source has been removed. It has already been shown that in fronds thus treated, no auxin can ever be obtained by diffusion. Similarly, it will be shown later in the present paper that such fronds do not continue to grow unless auxin is artificially supplied.

In view of the preliminary evidence presented above, it is clearly not possible to correlate diffusible and extractable auxin in the growing frond. Until a study of the disparity between the two has been made, it does not appear that the ether extraction method can contribute greatly to an understanding of growth processes in the frond.

Auxin and rachis elongation. It has been shown that of the considerable quantity of diffusible auxin which enters the leaf axis from the pinnae, a certain amount apparently disappears in the actively elongating portion of the rachis. It therefore seemed worth while to explore the relationship between the supply of diffusible auxin and the elongation of the rachis. This was done by excising varying numbers of pinnae from expanding fronds, while the fronds were still coiled and before any pinnae had been set off below the croziers (see Fig. 3).

Earlier experiments have shown that if all of the pinnae, including the terminal, are carefully excised from croziers several hours prior to diffusion, no diffusible auxin can be obtained from the bases of the resulting naked rachis. In such experiments, only a limited elongation occurs (Table VIII), and this is confined, for the most part, to the first 24-hour period or less. The values recorded in Table VIII refer to the region of the rachis which constituted the zone of elongation at the beginning of the experiment.

TABLE VIII

Effect of excision of pinnae, with or without added IAA, upon growth of zone of elongation

Treatment	Zone of elongation		
	Initial length, cm.	Final length, cm.	Per cent. elongation
Plant I			
Frond 1 Intact . . .	7·9	9·0	14
2 Pinnae removed . .	4·7	4·7	0
3 Pinnae removed + IAA	7·7	8·3	8
Plant II			
Frond 1 Intact . . .	10·4	13·5	30
2 Pinnae removed . .	9·7	10·1	4
3 Pinnae removed + IAA	11·6	15·0	29

In order to determine whether or not the removal of the auxin source is, in fact, responsible for the cessation of elongation in excision experiments, certain fronds were treated with an IAA-lanolin paste after the removal of the pinnae (Table VIII). It is evident that a partial, and sometimes nearly complete, recovery of elongation is obtained by the artificial introduction of auxin

into the system. It must be kept in mind that these figures pertain only to the zone of elongation at the time of pinna excision. The fronds as a whole do not, of course, continue their normal development.

An attempt was next made to correlate cell lengths in the ground tissue with observed elongation in the rachis in control and experimental plants. On individual plants one frond was left intact; one was cut about 90° into the crozier, so that all pinnae were removed, and a ring of lanolin was applied in the first 90°; and one was treated in this manner, but a ring of IAA in lanolin was used. At the beginning of the experiment a mark was placed on each frond at the base of the crozier, and the length of rachis from ground level to this mark was measured each day until no further increments were observed. Table IX indicates that the applied IAA brought about a partial recovery of elongation, although not as great as was observed in the previous series. At the outset of the experiment, the length of cells of the ground tissue in the first centimetre of rachis below the crozier was determined in one frond from each plant. At the conclusion of the study, similar cell measurements were made in the first centimetre of rachis below the mark in the remaining fronds. Since the cell measurements were made in a 1-cm. piece at the apex of the elongating zone only, and elongation occurred over a considerable length of rachis, one would not expect to find a very close correspondence in magnitude between overall growth and increase in cell size. Nevertheless, it is apparent from Table IX that the partial recovery of elongation under the influence of applied IAA is paralleled by a partial recovery of cell elongation in the ground tissue. Thus there is a correlation between rachis elongation, cell elongation, and the supply of diffusible auxin.

TABLE IX

Rachis elongation and cell length under the influence of applied and endogenous auxin

	Control	Intact	Pinnae removed	Pinnae removed + IAA
Initial cell size, μ	49·3	—	—	—
Final cell size, μ	—	143·0	58·3	89·9
Rachis increment, cm.	—	4·1	0·1	1·1

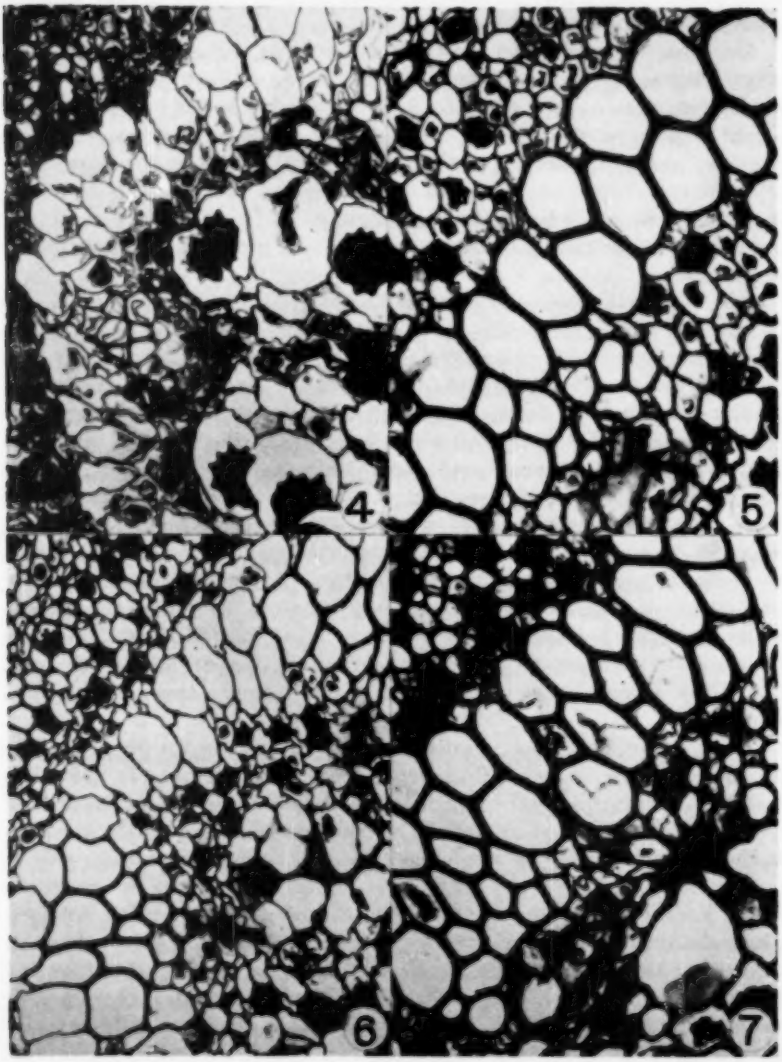
If the presence of pinnae is essential for normal rachis elongation because of the diffusible auxin which the pinnae contribute to the leaf axis, then removal of some of the pinnae should lead to a partial reduction in growth of the frond. This hypothesis was tested by excising various numbers of pinnae at a stage comparable to that described in previous experiments. Fig. 8 indicates that as many as 8 basal pairs of pinnae may be removed without a significant reduction in subsequent elongation of the frond. If 12 pairs are removed, some reduction is noted and more extensive excision leads to greater reductions in growth. These observations are somewhat surprising in view of the large proportion of laminar tissue removed when 8 pairs of pinnae are excised. Measurements made on a separate group of mature fronds have shown

that 8 basal pairs of pinnae account for nearly 50 per cent. by weight of the total laminar tissue.

One possible explanation for these observations is that the basal 8 pairs, since they are more fully developed at the time of excision than younger pinnae, do not account for a very large proportion of the total subsequent auxin production of the frond, although this would be contrary to findings reported earlier in this paper (Tables I and II). To test this possibility, diffusions were made from fronds from which various numbers of pinnae had been removed 18 hours earlier. Although the removal of 8 pairs does not produce a 50 per cent. reduction in auxin yield, it does lead to a significant diminution.

The relationship between auxin yield and growth in fronds with various numbers of pinnae removed is shown graphically in Fig. 8. It is apparent that auxin content of the frond may drop markedly without a significant reduction in total growth of the frond. When, however, the auxin content drops sufficiently, elongation is diminished, so that the earlier reported relationship between supply of diffusible auxin and growth is verified. It is of interest to note that the curves representing auxin yields above and below the zone of elongation in Fig. 8 remain essentially parallel as the auxin supply is decreased. This experiment was repeated on another occasion, with similar results. The apparent disappearance of diffusible auxin in the zone of elongation continues to about the same absolute extent even when the amount of auxin entering this zone is considerably reduced. It must be concluded from these observations that auxin produced by the pinnae is an essential factor in the normal elongation of the expanding frond. However, the amount of auxin which enters the rachis is considerably in excess of the minimum required for normal elongation.

Auxin and differentiation. In the experiments described in the preceding section (see Table IX), some further interesting observations were made. The leaf trace in the rachis of the frond of *O. cinnamomea* has somewhat the form of a broad, flattened horseshoe. At the beginning of the experiment the leaf trace in the outermost 90° of the crozier contained mature xylem tracheids only at a series of protoxylem points (25 or more) evenly spaced along the inner face of the horseshoe. The metaxylem region consisted of transversely enlarged procambial cells without evidence of secondary wall formation (Fig. 4). Seven days later, at the conclusion of the experiment, the treated and control fronds were examined histologically in the region which originally had been the outermost 90° of the crozier. Fig. 5 illustrates a portion of the vascular trace in this region of an intact normal frond. Xylem maturation is complete throughout the bundle, although differences in tracheid size indicate the original protoxylem points (arrows). By contrast, Fig. 6 illustrates the vascular strand of a frond from which the crozier above 90°, including all pinnae, had been removed and lanolin alone had been applied. Xylem differentiation has extended somewhat into the region which was originally procambial; but the bundle is clearly not completely mature. A portion of the



FIGS. 4–7. Transverse sections of portions of leaf traces. Protoxylem indicated by arrows. ×300. FIG. 4. In outermost 90° of uncoiling crozier. FIG. 5. Comparable region of intact frond 7 days later, after uncoiling and maturation. FIG. 6. Comparable to Fig. 5, but with crozier above first 90° excised at start of experiment. FIG. 7. Comparable to Fig. 6, but with IAA-lanolin paste applied at start of experiment. Details in text.

leaf trace of a frond to which IAA in lanolin had been applied following the excision is shown in Fig. 7. Without doubt, xylem maturation is as complete in this frond as it is in the normal frond (cf. Fig. 5). This experiment has been carried out three times with the same result. Thus, in addition to its role in promoting cellular elongation in the ground tissue, auxin appears to participate in some way in the final differentiation of xylem.

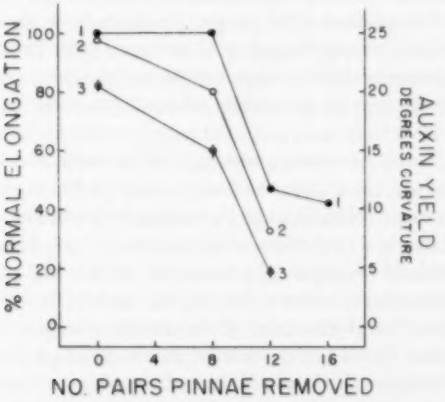


FIG. 8. Relationship between growth of frond and auxin yield above and below zone of elongation in expanding fronds, with various numbers of pinnae removed. Curve 1, per cent. of normal elongation. Curve 2, auxin yield above zone of elongation. Curve 3, auxin yield below zone of elongation. Each point in Curve 1 represents an average of measurements from 2 fronds. Auxin diffusions from pairs of comparable fronds.

Although the evidence is less distinct than in the case of xylem differentiation, there was also an influence of auxin upon the maturation of sclerenchymatous tissues of the hypodermal zone. At the beginning of the experiment no secondary walls could be observed in this tissue. At the conclusion of the study, the frond from which all pinnae had been removed showed no change in the hypodermis, while in the normal frond, extensive secondary wall formation and lignification had occurred. The application of auxin to a frond following excision of the crozier above 90° led to the formation of lignified secondary walls, although these were not as thick as those in the intact control frond.

DISCUSSION

The development of a foliage leaf of *O. cinnamomea* is a long and complex process involving a number of separate events which occur in an orderly sequence. Even the events of the fifth growing season with which this paper is concerned are brought about by an apparently complicated interaction between cell division and cell enlargement, and even, in the case of crozier uncoiling, a differential in extent of cell division in abaxial and adaxial ground

tissue of the rachis (Briggs and Steeves, 1958, 1959). Consequently, it might be anticipated that the problem of controlling mechanisms would be a difficult one to resolve. In the study reported here, only one of the many possible controlling factors, auxin, has been investigated. Such a study, although inadequate to provide a complete picture of the control of growth in this complex organ, is a necessary first step, and as such it is presented here.

The growth of the frond of *O. cinnamomea* differs in certain fundamental respects from leaves of most seed plants, particularly in its continuous acropetal developmental pattern (Briggs and Steeves, 1958). It might be expected, therefore, that the auxin relationships would have little in common with those studied in leaves of certain flowering plants by earlier workers, especially since previous studies have been confined largely to simple leaves. Comparison, however, reveals some interesting similarities, as well as certain differences. A number of reports have described net auxin yields from whole growing leaves; and in *Ipomoea* (Koning, 1933), *Solidago* (Goodwin, 1937), and *Aster* (Delisle, 1938) there is a rise in auxin production to a maximum followed by a decline and ultimate cessation of production as the leaf matures, just as in *Osmunda*. Furthermore, Goodwin (1937) has shown that the peak of auxin production occurs close to the time of maximum elongation rate of the leaf, again as in *Osmunda*. However, the auxin peak in *Solidago* precedes maximum elongation rate, whereas in *Osmunda* the relationship is reversed.

In the developing tobacco leaf Avery (1935) observed that auxin is produced rather uniformly throughout the lamina and moves into the main lateral veins and the midrib, where it accumulates. Transport in the veins is polar, and auxin accumulates in the proximal portion of the leaf. Avery has suggested that its presence in high concentration is correlated with continued growth in that region. It is, of course, difficult to make comparisons between the pinnate frond of *Osmunda* and the simple leaf of tobacco, but production of auxin in the pinnae and its subsequent movement into the rachis where transport is in a basipetal direction is certainly suggestive of Avery's findings. On the other hand, except for cases of an apparent failure of the transport system in the leaf base, auxin does not accumulate in the proximal portion of the *Osmunda* leaf, and in any event, it is not correlated with any developmental phenomena in the leaf base. Thus, the overall pattern of auxin production in *Osmunda* fronds agrees with that of leaves of flowering plants as do certain general features of development, such as the sigmoid growth curve and ultimate complete maturation of the leaf. Moreover, just as the internal localization of growth differs in the two leaf types, so does the internal distribution of auxin.

It has been shown that two distinct morphogenetic processes in the rachis, elongation and the final differentiation of xylem and probably also of the sclerenchymatous hypodermal zone, require a supply of diffusible auxin for their normal completion. To these must be added a third, crozier uncoiling, which has been shown elsewhere (Briggs and Steeves, 1959) to be dependent upon auxin normally provided by the pinnae. The promotion of elongation

involves, as far as can be determined, a direct influence upon the enlargement of ground tissue cells without any participation of cell division. The contribution of auxin to crozier uncoiling is not basically different. The mechanism of crozier uncoiling involves more extensive cell division in the adaxial ground tissue of the coiled rachis than in the abaxial region, which equalizes an inequality in cell number established during crozier formation. As a result, the cells in the adaxial tissue are smaller than those in the abaxial when enlargement of cells in both regions begins. The smaller cells of the adaxial region elongate more extensively and overtake those of the abaxial region so that uncoiling occurs. It is apparent that auxin plays no part in the equalizing of cell numbers; but it is essential for the elongation which brings about the actual uncoiling.

The dependence of xylem differentiation upon a supply of diffusible auxin is perhaps not surprising in view of earlier reports of the influence of auxin upon this process in wounded stems of *Coleus* (Jacobs, 1952), in cultured pea roots (Torrey, 1953), and in callus tissue cultures (Camus, 1949; Wetmore and Sorokin, 1955). Recently, Jensen (1955) has provided evidence for the promoting influence of IAA upon lignification of vascular tissues through an induction of peroxidase formation. The present study has dealt only with the final stages of xylem maturation, namely, secondary wall formation and lignification, and no information is at present available as to the earlier stages of differentiation.

In the three morphogenetic phenomena which have been discussed, auxin, while it is essential, can hardly be considered as a determining factor. In the early stages of leaf development during the final growing season, cellular elongation is confined entirely to the region below the crozier. At a later stage, correlated with the onset and acceleration of uncoiling, limited cellular elongation extends into the outer coil of the crozier. Auxin directly promotes this elongation, yet it is clear that rachis tissue within the crozier and above the elongating region receives a plentiful supply of auxin but does not elongate significantly. Moreover, below the zone of elongation, growth stops, while a considerable, though reduced, amount of diffusible auxin is still present. Furthermore, it has been shown that, at least during part of the final expansion, the amount of auxin present is in excess of the minimum required for normal elongation or for uncoiling. Auxin is continuously present, although the quantities vary somewhat. The acquisition of what may be called competence to elongate (Briggs and Steeves, 1959) and the ultimate cessation of elongation must be determined by other, as yet unknown factors. In the case of final differentiation of xylem and hypodermal sclerenchyma, the reaction to auxin occurs only in certain cells and at a certain stage in their development; and all other cells are insensitive. In all three of these phenomena, auxin must be considered an essential participant, but not a specific determining factor, since the response which it evokes depends upon the physiological states of the cells involved.

When diffusible auxin is assayed, a picture emerges in which auxin moves

through immature regions which are not elongating, and through mature regions, without significant disappearance. On the other hand, in elongating tissue the concentration drops markedly so that there is a clear correlation between auxin disappearance and growth by cell elongation. In the fronds in which this study was made, the elongating region was below the crozier. It is possible that when, in later stages of development, cellular elongation extends to a limited extent into the crozier, there is a drop in auxin content in the outermost part of the crozier. However, at this time, crozier uncoiling is in progress, and the presence of auxin-producing pinnae on the rachis in the outer half turn of crozier makes it extremely difficult to approach this problem. Disappearance of auxin in relation to growth is by no means a new phenomenon, and it was described on a quantitative basis in the *Avena* coleoptile by Bonner and Thimann as early as 1935. It has been reported or indirectly alluded to repeatedly since that time.

Bonner and Thimann (1935) thought of the disappearance as resulting from a transformation of auxin which was essential for its participation in the growth process. However, in recent years there has been a strong tendency to regard auxin participation in growth as of a catalytic nature (Thimann, 1952), in which case the auxin should emerge from the growing region qualitatively and quantitatively unchanged. It no longer seems reasonable to account for auxin disappearance as a manifestation of 'utilization' in the growth process. Yet these concepts must be reconciled with clear evidence for a correlation between auxin disappearance and growth.

One possible explanation for the apparent disappearance of auxin in the zone of elongation might be the participation of an enzymatic auxin destruction system (Ray, 1958). In order to account for the distribution of auxin as determined by diffusion on this basis, it is necessary to assume that such a system is only active in the zone of elongation, and not in either the crozier or the zone of maturation. However, it has been shown that consistent yields of auxin cannot be obtained by diffusion at any level of the rachis except in the presence of potassium cyanide (Steeves *et al.*, 1953). Thus there is no reason to suppose that such a destruction system is confined to the zone of elongation, although it is known to be more active in extracts of immature regions than of mature (Briggs *et al.*, 1955*a*). Moreover, it has been shown that the drop in diffusible auxin in the zone of elongation is relatively constant regardless of the amount of auxin entering this region (see Fig. 8). Unless such an enzyme system were limiting, the quantity of auxin which disappears should diminish as the amount entering the zone of elongation diminishes. These observations, together with previously published studies on auxin destruction in *O. cinnamomea*, make it extremely unlikely that auxin inactivation *in vivo* accounts for the consistent drop in the zone of elongation.

Ether extractions have revealed no drop in concentration in the zone of elongation such as is apparent in diffusion studies. Although caution must be exercised in comparing these two types of data, because it is not certain that the concentrations determined by extraction are validly expressed, the differ-

ence suggests another possible explanation for apparent disappearance of auxin. Diffusion can measure only auxin which is actually moving in the transport system. It is at least possible that, in its involvement in the growth process, auxin is withdrawn from the transport system; and there might well be a delay, or even a failure, in its re-entry into the system after its involvement. It could also be bound in some way, but in this event it would not be expected to be extracted by short-term exposure to ether at low temperatures (van Overbeek *et al.*, 1947). This interpretation might also provide an explanation for the retention of auxin for as much as 48 hours after the removal of the auxin source, in certain cases. If auxin were present but not in the transport system, it might be expected to remain within the tissue for a considerable period of time, assuming that it is not inactivated.

Evidence has been provided that, in the *Osmunda* frond diffusible and ether-extractable auxin cannot be equated. The apparent disappearance of diffusible auxin is closely correlated with growth, and removal of the auxin source invariably causes growth to cease even though extractable auxin may be present. It may be concluded, therefore, that diffusible auxin is more closely associated with growth phenomena than is ether-extractable auxin. This idea might be extended to indicate that only auxin which is moving in the transport system is available to the growth process. If, as suggested above, auxin is withdrawn from the transport system in growth, then it must be effective only immediately after its withdrawal since auxin present in the tissue but not moving in the transport stream seems to be incapable of promoting growth. In any event it is clear that, until more evidence is available, great caution must be exercised in interpreting growth phenomena on the basis of ether-extractable auxin in this species.

A final problem to be considered is the failure to obtain auxin by diffusion from the leaf base on many occasions. The complete or partial failure of the auxin transport system would seem to be the most likely explanation for these results, since extraction reveals, not only abundant auxin in this tissue, but a distinct tendency for an accumulation. It has been shown previously (Briggs and Steeves, 1958) that the leaf base is essentially mature at the end of the fourth growing season, while the aerial portion of the rachis does not reach maturity until the early part of the fifth. It is at least possible that the failure of auxin transport could be associated with the greater age of the tissue involved. Since considerable variability occurs, such a change must be correlated with physiological changes associated with ageing, rather than with absolute age. Since it is important to know how much auxin normally enters the rhizome from the leaves, and under what conditions, this problem is currently under investigation. It will be considered at a later time in connexion with a study of auxin relationships in the rhizome.

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LITERATURE CITED

ALBAUM, H. G. (1938). Inhibitions due to growth hormones in fern prothallia and sporophytes. *Amer. J. Bot.* **25,** 124–33.

ALLSOPP, A. (1954). A comparison of the effects of 3-indolylacetic acid and 3-indolylacetonitrile on the development of sporelings of *Marsilea* in aseptic culture. *J. Exp. Bot.* **5,** 16–23.

—— (1956). Morphogenetic effects of 3-indolylacetonitrile on sporelings of *Marsilea* in aseptic culture. Ibid. **7,** 1–13.

AVERY, G. S. (1935). Differential distribution of a phytohormone in the developing leaf of *Nicotiana*, and its relation to polarized growth. *Bull. Torrey Bot. Club*, **62,** 313–30.

BONNER, J., and THIMANN, K. V. (1935). Studies on the growth hormone of plants. 7. The fate of growth substance in the plant and the nature of the growth process. *J. Gen. Physiol.* **18,** 649–58.

BRIGGS, W. R., and STEEVES, T. A. (1958). Morphogenetic studies on *Osmunda cinnamomea* L.: The expansion and maturation of vegetative fronds. *Phytomorphology*, **8,** 234–48.

—— —— (1959). Morphogenetic studies on *Osmunda cinnamomea* L.: The mechanism of crozier uncoiling. Ibid. (in press).

——, MOREL, G., STEEVES, T. A., SUSSEX, I. M., and WETMORE, R. H. (1955*a*). Enzymatic auxin inactivation by extracts of the fern *Osmunda cinnamomea* L. *Plant Physiol.* **30,** 143–8.

——, STEEVES, T. A., SUSSEX, I. M., and WETMORE, R. H. (1955*b*). A comparison of auxin destruction by tissue extracts and intact tissues of the fern *Osmunda cinnamomea* L. Ibid. 148–55.

CAMUS, G. (1949). Recherches sur le rôle des bourgeons dans les phenomènones de morphogénèse. *Revue Cytol. Biol. Végét.* **11,** 1–199.

DELISLE, A. L. (1938). Morphogenetical studies in the development of successive leaves in *Aster*, with respect to relative growth, cellular differentiation, and auxin relationships. *Amer. J. Bot.* **25,** 420–30.

DU BUY, H. G., and NUERNBERGK, E. L. (1938). Growth, tropisms and other movements. In *Manual of Pteridology*. Ed. by Fr. Verdoorn. Pp. 303–46. Martinus Nijhoff. The Hague.

GOODWIN, R. H. (1937). The role of auxin in leaf development in *Solidago* species. *Amer. J. Bot.* **24,** 43–51.

JACOBS, WM. P. (1952). The role of auxin in differentiation of xylem around a wound. Ibid. **39,** 301–9.

JENSEN, W. A. (1955). The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. *Plant Physiol.* **30,** 426–32.

KONING, H. C. (1933). Het winden der slingerplanten. Diss. Utrecht.

PRANKERD, T. L. (1922). On the irritability of the fronds of *Asplenium bulbiferum*, with special reference to graviperception. *Proc. Roy. Soc. London*, **93,** 143–52.

RAY, P. M. (1958). Destruction of auxin. *Ann. Rev. Plant Physiol.* **9,** 81–118.

STEEVES, T. A., and BRIGGS, W. R. (1958). Morphogenetic studies on *Osmunda cinnamomea* L. The origin and early development of vegetative fronds. *Phytomorphology*, **8,** 60–72.

—— and WETMORE, R. H. (1953). Morphogenetic studies on *Osmunda cinnamomea* L.: Some aspects of the general morphology. Ibid. **3,** 339–54.

——, MOREL, G., and WETMORE, R. H. (1953). A technique for preventing inactivation at the cut surface in auxin diffusion studies. *Amer. J. Bot.* **40,** 534–8.

THIMANN, K. V. (1952). Plant growth hormones. In *The Action of Hormones in Plants and Invertebrates*. Ed. by K. V. Thimann. Pp. 1–76. Academic Press. New York.

TORREY, J. G. (1953). The effect of certain metabolic inhibitors on vascular tissue differentiation in isolated pea roots. *Amer. J. Bot.* **40,** 525–33.

VAN OVERBEEK, J., DE VÁZQUEZ, E. S., and GORDON, S. A. (1947). Free and bound auxin in the vegetative pineapple plant. Ibid. **34**, 266–70.

WARDLAW, C. W. (1946). Experimental and analytical studies of pteridophytes. 8. Further observations on bud development in *Matteuccia struthiopteris*, *Onoclea sensibilis*, and species of *Dryopteris*. *Ann. Bot.* N.S. **10**, 117–32.

WETMORE, R. H., and PRATT, C. (1949). The growth and auxin relationships of leaves of the maidenhair fern, *Adiantum pedatum* L. (Abstract). *Amer. J. Bot.* **36**, 806.

—— and SOROKIN, S. (1955). On the differentiation of xylem. *Jour. Arnold Arboretum*, **36**, 305–17

WENT, F. W., and THIMANN, K. V. (1937). *Phytohormones*. Macmillan Co. New York.

Cell Division and Expansion in the Growth of the Leaf

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SUMMARY

Volumes and numbers of cells were determined at different stages of development of the fifth leaf of *Lupinus albus*, and each of the second pair and the tenth leaf of *Helianthus annuus*. In the case of the second pair of sunflower leaves the values cover the whole life of the leaf from initiation to senescence.

During both primordial development and the ensuing 'grand period of growth' division is the determinant of growth. About 10 per cent. of the cells in the fully grown leaf are laid down before leaf-emergence; the remaining 90 per cent. are formed during unfolding. Division does not cease in the lupin leaf or the second pair of sunflower leaves until they have reached half their maximum area. The tenth leaf, on the other hand, is as much as three-quarters fully grown before division ceases.

Cell expansion commences soon after leaf initiation and continues throughout the life of the leaf. With lupin and the second pair of sunflower leaves there is a fourfold increase in the average volume of the cells before emergence from the apical region. During unfolding, there is a further tenfold increase in the average volume of the cells of the lupin leaf, and a twentyfold increase with the second pair of sunflower leaves. Expansion continues after the cessation of division but this further increase in volume is comparatively small.

The data are discussed in relation to the 'two phase' hypothesis of leaf development.

INTRODUCTION

THE purpose of the investigation, the results of which are presented below, is the interpretation of leaf growth in terms of the two fundamental growth processes of cell division and cell expansion. The data illustrate the changes which occur in the number of cells and in the average volume which they occupy from an early stage in the initiation of the leaf at the apex of the shoot to a stage when it is fully mature and has passed over into senescence.

In the past, leaf growth has been extensively investigated mainly in terms of increases in linear dimensions, changes of shape or area, but the contributions of division and expansion are as yet little understood. In the absence of quantitative evidence, however, it is often stated that the two processes determine leaf growth in two distinct developmental phases. It has been suggested that during the primordial and early life of the leaf, growth is almost if not wholly due to the formation of new cells; and that division ceases early, subsequent growth being the expression of cell expansion (papers in *The Growth of Leaves*, Milthorpe, 1956).

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Where attempts have been made specifically to interpret the size of the leaf in terms of cellular conditions, reference has been made only to changes in number and area of the epidermal cells (von Papen, 1935; Ashby and Wangermann, 1950). The latter authors, for example, showed that division in the epidermis of the *Ipomoea* leaf goes on until it is about 5 per cent. of its fully developed area. From then onwards there is very little division, growth being determined wholly by expansion. There is no increase in epidermal cell size until division ceases.

It is evident, however, that the duration of division in the epidermis differs considerably from that in the other tissues of the leaf. In the tobacco leaf division ceases first in the epidermis and continues for a longer period in the internal tissues (Avery, 1933). It is therefore probable that changes in the epidermis do not correspond to changes in the whole leaf.

The ultimate objective, it is true, is the expression of the changes in the number and size of every cell type within the developing leaf, but in the first place a considerable amount of information may be obtained from a consideration of the cell population as a whole. In the present investigation, therefore, discussion is restricted to changes in the total number of cells and these are used to indicate the overall contribution which division makes to growth.

With respect to size, it is the volume of the cells rather than their area which is likely to be the best measure of cell expansion. Here, estimates of the total volume of the cell population have been made and from these values and the total numbers of cells an average value for the volume of the cells at different stages of leaf development has been calculated. The changes in average cell volume are used as an indication of the overall contribution of expansion.

The use of changing epidermal cell area for investigations of leaf growth has a further disadvantage. The technique tends to limit measurements to the later stages of growth. Conditions within the leaf primordium are, however, of decisive importance in a consideration of the development of the whole leaf. Recently techniques for determining total cell numbers and volumes of leaf primordia have been described by Sunderland and Brown (1956). Determinations were made on the first seven leaf primordia of seedlings of *Lupinus albus* and it was shown that the average volume of the cells increased about threefold from the youngest to the oldest primordium. These results, although they do not refer to the growth of a single leaf primordium, nevertheless show that cell expansion makes a significant contribution even in the earliest stages of development.

MATERIALS AND METHODS

Leaves of two species, *Lupinus albus* and *Helianthus annuus*, have been used. In the case of the lupin observations have been made on the fifth leaf above the cotyledons. The primordium of the fifth leaf is embryonic in the sense that it is one of six on the plumule of the seed.

With sunflower, on the other hand, the data refer to leaves which have

been initiated after germination. Two series of determinations have been made, one on the second pair of leaves and the other on the tenth leaf. The second pair, which arise simultaneously at right angles to the first, are the first primordia to appear after germination has commenced. The initiation of primordia in pairs does not, however, continue, and with further development the phyllotaxis changes from a decussate to an alternate arrangement. This is fully established before leaf 10 is initiated. To avoid confusion, therefore, the second pair of leaves has been treated as one, and the values given below are the means obtained from estimations of both leaves of the pair. Ten determinations have been made on each sampling occasion.

The leaves or primordia have been removed from seedlings selected at random from one bulk sowing. The seeds have been planted in boxes and allowed to germinate at a temperature of 12·5–15·5° C. After about 30 days the seedlings have been potted individually and left under ordinary glasshouse conditions.

In addition to cell number and volume, measurements of length and area have been made. The length of primordial leaves has been taken from the base to the apex by measurement against an ocular micrometer. In the unfolding condition length has been measured from the base of the petiole to the tip of the leaf. Area has been determined from tracings.

Primordial stage. Determinations of cell number and total volume have been made by the methods described by Sunderland and Brown (1956).

The operations involved in the dissection of the primordia from the plumule have also been based on the methods of these workers. From the selected seeds the testa is removed and one or both of the cotyledons is cut away to expose the embryo. With lupin both cotyledons are removed because the radicle is sufficiently large to enable the embryo to be gripped by the operator. In the case of sunflower, the radicle is small in the initial stages and it is easier to hold the embryo with one of the cotyledons.

The removal of the cotyledons reveals the plumule bearing primordia surrounding the apical meristem. With lupin four primordia are removed, starting with the oldest, until the fifth is reached. Primordia are most easily removed when sufficiently well developed by slitting along the base with the point of dissecting tweezers. The whole primordium with its accompanying stipules may then be gently peeled away.

In the earliest stages when the primordium is comparatively undifferentiated it is not possible to remove it in this way. Instead the primordium is removed from the plumule attached to part of the apical meristem. The whole apical region bearing the fifth and younger primordia is excised from the seed on a column of tissue by four cuts made longitudinally downwards into the hypocotyl. The strip of tissue thus isolated is then transferred by forceps to a pad of moistened potters' clay into which the strip of damaged tissue is pressed.

The apex is arranged during embedding in such a way that the base of the fifth leaf is clearly visible. A cut is made through the base of the leaf at right angles to the long axis, thus isolating a fragment of tissue bearing the apical

meristem plus the leaf primordia. This is transferred from a horizontal to a vertical position with the cut surface resting on the clay, and the primordium is removed from the isolated fragment by means of a vertical cut in the angle between the axis and the primordium.

Dissection and excision of the sunflower primordia have been carried out in a manner similar to that described above for the lupin.

All the operations have been done under a dissecting microscope at a magnification of about 70×. Fine watchmaker's tweezers have been used (Dumont Fils No. 5) and the cuts have been made by means of small pieces of razor blade held in sliding tongs.

Of the values given below, those recorded for lupin after 2, 4, 6, and 8 days and for sunflower after 9, 13, 17, and 21 days refer to the methods described above. In all subsequent determinations measurements of volume and cell number have been carried out as outlined in the next section.

Unfolding and mature stages. As an estimate of the total volume of the cells fresh weight has been used. This is preferable to the total volume of the leaf since this is likely to be affected considerably by the formation of air spaces, and it is the volume of the cells rather than the leaf volume with which the investigation is concerned. Values have been recorded in milligrams (assuming 1 $mm.^3 = 1$ mg.).

The technique for cell-number estimation has been adapted from that described by Brown and Rickless for root tissues (1949). Each leaf or pair of leaves is immersed in a known volume of 5 per cent. chromic acid solution and is left immersed at room temperature for a standard time. After treatment with the acid solution, the tissue is broken up into a fine slurry by manipulating it with a thin glass rod against the sides of the vessel. The slurry of cells is mixed with the fluid and the final suspension, on which the count is made, is obtained by squirting the fluid rapidly several times through the narrow orifice of a pipette.

The proportion of acid solution to amount of leaf tissue is extremely important. With young leaves up to about 2 cm. in length 10 ml. of the solution is adequate. With increasing size, however, it is necessary to use more chromic acid. A leaf of 50 sq. cm. may require as much as 200 ml. of macerating fluid. Infiltration under reduced pressure is also necessary with older leaves.

Time of immersion in chromic acid is critical, and this varies with the age of the leaf. Prolonged exposure to the acid causes disintegration of some of the cells. With young expanding leaves this danger may be disregarded and they may be left in the acid for as long as 96 hours. With increasing maturity and progressive enlargement of the cells they become more susceptible to damage and it is necessary to reduce the time of immersion.

In this investigation a maximum of 4 $cm.^2$ of leaf has been used to each 10 ml. of chromic acid solution. In the case of sunflower, the second pair of leaves has been exposed to the acid solution for 24 hours up to the 35th day from sowing. After that, they have been left for a maximum of 12 hours. All the values for the tenth leaf refer to 12-hour periods of immersion.

It was found that the lupin leaves are more difficult to manipulate than sunflower and 24 hours' maceration at least was necessary to yield an adequate cell suspension. With increasing age, however, the leaves become more difficult to macerate even after long periods of immersion. Consequently observations on the lupin leaf were discontinued before the mature size was reached.

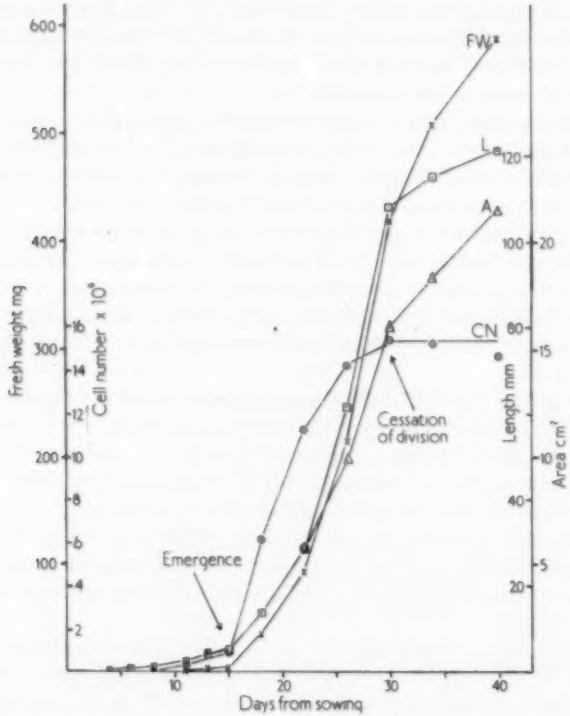

FIG. 1. Fresh weight *FW*, cell number *CN*, length *L*, and area *A* of the fifth lupin leaf.

EXPERIMENTAL RESULTS

The data are assembled in Figs. 1, 2, and 3. For each leaf changes in length, area, total fresh weight or volume, and total number of cells are shown. Fig. 1 refers to the fifth lupin leaf; Fig. 2 to each of the second pair of sunflower leaves and Fig. 3 to the tenth sunflower leaf. The average cell volumes are shown on Fig. 4.

It is evident that the growth curves for each leaf follow very similar courses. Growth-rate in the early stages of development is comparatively low. This early phase is then followed by one of very marked increases corresponding to the 'grand period of growth' of Sachs. With further development the size

of the leaf continues to increase gradually but again at a much lower rate until ultimately the leaf senesces.

The overall cellular conditions in each of these three stages of development are now described. It should be emphasized, however, that this procedure is adopted to facilitate description of the data rather than to indicate that leaf growth occurs in three distinct phases.

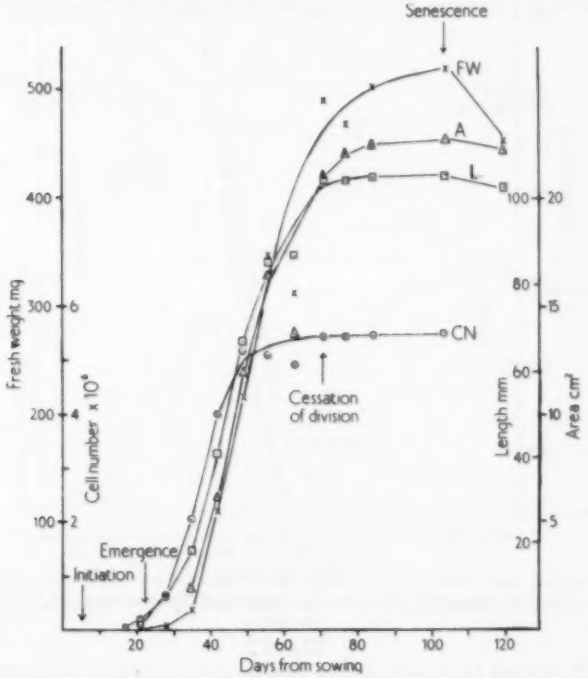

FIG. 2. Values for each of the second pair of sunflower leaves. Symbols as in Fig. 1.

Pre-emergence or primordial development. As indicated above, absolute growth of the primordia is comparatively low and because of the difficulty of recording the low values accurately on the figures they have been assembled in Table I.

From these values it is evident that during the first 15 days there is a 1,440-fold increase in the volume of the lupin primordium and this is accompanied by a 340-fold increment in cell number. In the case of the second pair of sunflower leaves, from the 9th to the 21st day there is a 325-fold increase in total volume and a 100-fold increase in cell number. In both cases the average volumes of the cells increase about fourfold, and it is clear that enlargement of the primordium is due partly to the formation of new cells and partly to the fourfold increase in their average volume.

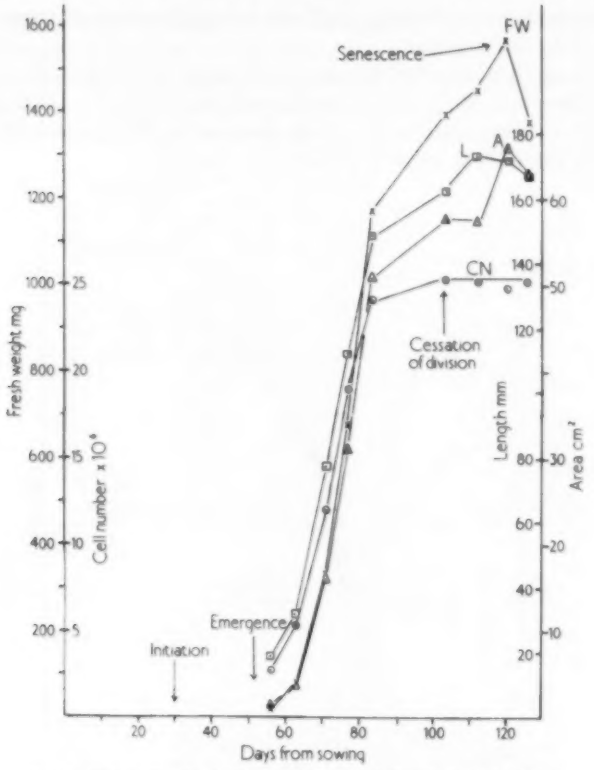

FIG. 3. Values for the tenth sunflower leaf. Symbols as in Fig. 1.

TABLE I

Values for the fifth leaf of lupin and each of the second pair of sunflower leaves

Length L (mm.); number of cells N; total volume V (mm.³); fresh weight FW (mg.); and average cell volume CV (mm.³ × 10^{-7})

Days from sowing	L	N	V	FW	CV
		Lupin			
2	—	2,730	0·0025	—	9·2
4	0·33	8,000	0·0076	—	9·5
6	0·6	24,500	0·023	—	9·4
8	1·1	74,600	0·13	—	17·4
11	2·4	252,000	—	0·81	32·1
13	4·7	748,500	—	3·3	44·1
15	5·1	920,500	—	3·6	39·1
		Sunflower			
9	—	920	0·0012	—	13·0
13	—	3,300	0·0035	—	10·6
17	1·05	15,100	0·056	—	37·1
21	2·4	90,300	0·39	—	43·2

Emergence and unfolding of the leaf. The period of maximum growth of the fifth lupin leaf is indicated in Fig. 1 by the data from 15 to 40 days after sowing. In the case of sunflower, the corresponding period in Fig. 2 for the second pair of leaves is from 21 to 71 days and in Fig. 3 for the tenth leaf from 56 to 104 days.

In each case unfolding of the leaf is marked by large increases in fresh weight. In the case of the lupin, fresh weight increases from 3·6 to about 590

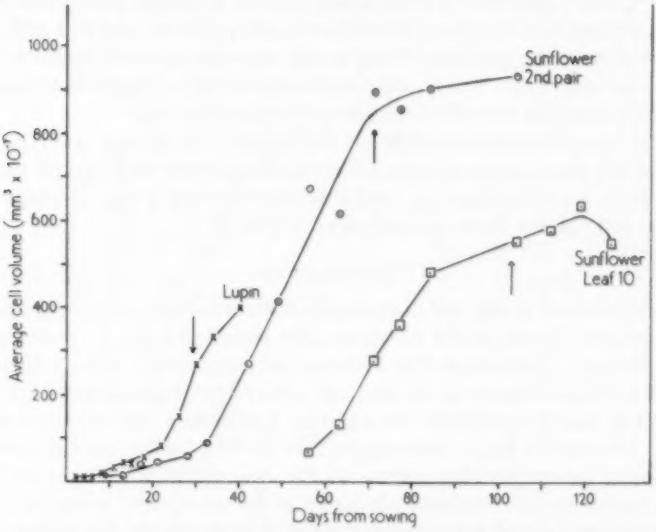

FIG. 4. Changes in average cell volume during growth of the fifth lupin leaf, each of the second pair of sunflower leaves, and the tenth sunflower leaf. The arrow denotes cessation of division.

mg. when the leaf is then 21 cm. in area and 122 mm. in length. The fresh weight of each of the second pair of sunflower leaves during the period from 21 to 71 days increases from 0·39 to 490 mg. and the leaf is then 21 cm.^{2} in area and 105 mm. long. The tenth sunflower leaf is larger than the second pair and by the 104th day it is 163 mm. in length with an area of about 58 cm.^{2} During unfolding the fresh weight of the tenth leaf increases from a value of about 18 mg. just after emergence to 1,400 mg. on the 104th day.

It is most significant that the large increases in fresh weight are accompanied by the formation of large numbers of cells. By the 30th day the number of cells of the fifth lupin leaf has increased from 920,000 to approximately 15 millions. The number of cells in the second pair of sunflower leaves increases to 5·5 millions and in the tenth leaf to about 25 millions.

Clearly, during the grand period of growth, both division and expansion contribute to the enlargement of the leaf. The resultant effect of the two processes causes a considerable increment in the average cell volume. With the

lupin leaf, the increase is about tenfold, and with the second pair of leaves and the tenth sunflower leaf the increases are twentyfold and sevenfold respectively.

Maturation and senescence. The leaf continues to enlarge after the cessation of division. In Fig. 2 it may be observed that the fresh weight of the second pair of sunflower leaves continues to change after the 71st day. It increases slightly from 490 to 520 mg. by the 104th day and then falls with the onset of senescence. The increase in fresh weight after the cessation of division is, however, small and there is little change in area or length during this period.

It is evident that the peak formation of cells in the second pair of leaves is over by at least the 49th day. Even so, the increase in fresh weight from the 49th to the 104th day is only about twofold and the average cell volume is at most only doubled after the formation of most of the cells.

In the case of the tenth sunflower leaf division is not over until the 104th day. During the ensuing 22 days before senescence the fresh weight increases from about 1,400 to 1,600 mg. and this involves only a slight change in the average cell volume from 550 to 630 $mm.^3 \times 10^{-7}$.

DISCUSSION

Cell formation in the leaf is evidently not restricted to the early stages of development. It goes on for a considerable period of time. From the data of Fig. 2 it may be calculated that about 90 per cent. of the cells of the second pair of sunflower leaves are formed when they have reached half their maximum area, and it is probable that a further half million cells are subsequently added. The data of Fig. 1 also suggest that the fifth leaf of lupin is more than half grown before division ceases. In the case of the tenth sunflower leaf, which has an area almost three times that of the second leaf, division does not apparently cease until it has reached more than three-quarters its full size.

It is also evident that the bulk of the cells are formed, not in the primordium but after emergence when the leaf is unfolding. With lupin about 14 of the 15 million cells, and in the case of the tenth sunflower leaf, 23 of the 25 million cells which constitute the mature leaf are formed after emergence. In terms of absolute growth therefore cell formation is greatest during unfolding of the leaf.

The formation of only 10 per cent. of the cells during primordial growth is not indicative of a low relative rate. This is shown by the data of Figs. 5 and 6 in which logarithmic values of the data of Fig. 1 and 2 are plotted. The relative increase in cell number for both lupin and sunflower leaves is greatest in the early stages of growth. It gradually falls as the leaves develop and is unaffected by leaf emergence.

With the fifth lupin leaf about 920,000 cells are formed before emergence and these are formed from a primordial mass which 2 days after sowing consists of 2,700 cells. During the first 15 days, therefore, something like 8 to 9 generations of cells are formed indicating an average rate of division of one generation in slightly under 2 days. The formation of the remaining 14 million cells involves another 4 generations and these are produced

during the next 15 days at an average rate of one generation in approximately every 4 days.

In the case of the second pair of sunflower leaves 90,000 cells are formed in 16 days from a primordium which 4 days from initiation consists of 900 cells. Thus in the early stages of growth before emergence 3 to 4 generations of cells

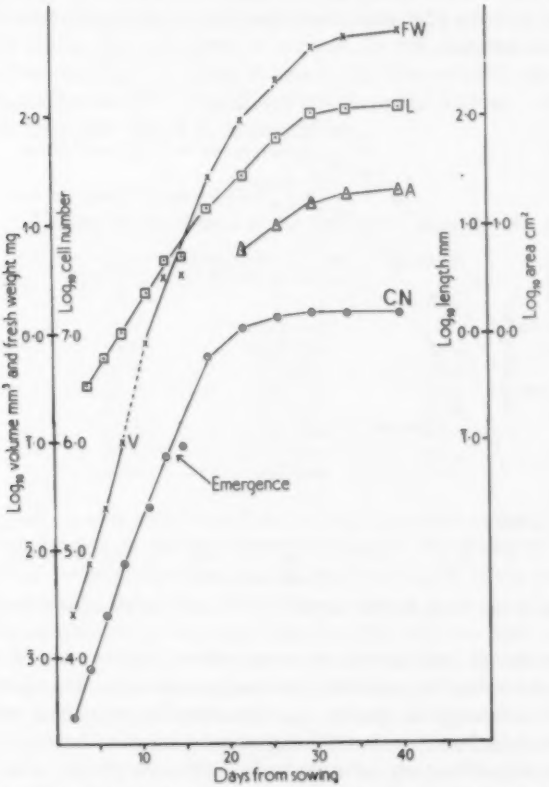

FIG. 5. Log_{10} values for volume *V*, fresh weight *FW*, cell number *CN*, length *L*, and area *A* of the fifth lupin leaf.

are produced at an average rate of one in every 3 to 4 days. The formation of the remaining cells, after emergence, involves a further 6 to 7 generations. If it is assumed that one generation of cells has been already formed before the measurements commenced, a total of 13 to 14 generations is involved in the growth of the lupin leaf, and 11 to 12 in the second pair of sunflower leaves.

The addition of one or more generations of cells during unfolding will affect considerably the ultimate size of the leaf and in all probability this is the

case with the tenth leaf of sunflower. In Fig. 3 it is evident that the life of the tenth leaf occupies about 100 days. Division proceeds until the 76th day after initiation. This is in contrast to the second pair of leaves where the bulk of the cells are formed by the 44th day after initiation. The greater number of cells in the tenth leaf arise, therefore, at least in part because of the longer duration of division, and the data suggest that division not only proceeds for a considerable part of the life of the leaf, but that its duration varies from leaf to leaf on the same stem.

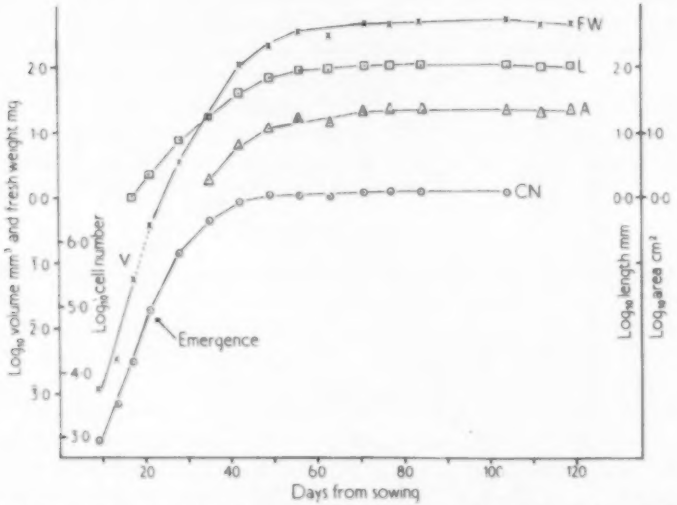

FIG. 6. Log_{10} values for each of the second pair of sunflower leaves. Symbols as in Fig. 5.

The concept of leaf growth as a two-phase system, one dominated by division and another by expansion, is clearly untenable. The developing leaf, like any other biological system, is a dynamically expanding unit in which cells are continually formed and subsequently expand. At any stage the leaf consists of cells of varying volumes and in different phases of development.

The contribution of cell expansion in leaf growth is readily assessed from the data of Figs. 5 and 6. The relative increase in the total volume is greatest during primordial development and it decreases with time. The shapes of the curves for fresh weight, length, or area closely follow those for cell number. It may be emphasized that there is no break to suggest a sudden or marked change in the development of the system. The curves for fresh weight and cell number differ in one respect only, that is, in the angle of slope. The curve for fresh weight is steeper than that for cell number and it is so throughout its course. Thus, when division ceases, expansion continues and the final maturation of the leaf in this sense may be interpreted as due to the expansion of the last-formed cells.

The progressive change in the average cell volume (Fig. 4) which arises as a result of the difference in the relative rates of growth as measured by division and expansion is a reflection of the continually expanding population. Expansion commences soon after leaf initiation and continues throughout the entire development of the leaf. The cell volume of the lupin leaf increases fortyfold over the period of growth investigated, and with the second pair of sunflower leaves the overall increment is as high as ninetyfold. There can be little doubt that the greatest increase in cell volume occurs during unfolding of the leaf but it is again stressed that this period is also the one of greatest cell formation. The overall increment in the average volume of the cells after division has ceased is extremely low.

TABLE II

Values for the petiole of the tenth leaf of sunflower

(Symbols and quantities as in Table I)

Days from sowing	L	N	FW	CV
63	3·9	241,900	11·1	459
71	14·6	478,500	45·4	949
77	26·0	761,300	100·0	1,313
84	38·3	905,000	186·7	2,063
104	45·6	1,051,300	241·0	2,293
112	52·5	1,031,300	267·0	2,590
119	46·3	1,007,810	211·4	2,098
126	47·6	996,900	181·3	1,819

The conclusions with respect to division and expansion in the growth of the whole leaf apply also to the growth of the petiole. In Table II values are shown for fresh weight, numbers of cells, and the length of the petiole of the tenth sunflower leaf. The observations cover a period from the stage when the petiole is distinguishable as a morphological entity until the death of the leaf.

The data show that the petiole extends to a length of 48 mm. in 63 days; the fresh weight increases about sixteenfold. The extension of the petiole involves a fivefold increase in the average volume of the cells but it is clear that this is not wholly due to expansion. There is a fourfold increase in the number of cells. Division ceases in the petiole on the 74th day after initiation which is the time when division ceases throughout the entire leaf.

One further point is emphasized by the data of Fig. 4. The average cell volumes of the sunflower leaves differ considerably. The average volume of the cells of the tenth leaf is much less than that of the second pair of leaves. Nevertheless, the tenth leaf is the larger of the two. Differences in size of epidermal cells of successive leaves on the same plant have often been commented on in the past, and it is now apparent that the average volume of the whole cell population differs likewise.

Differences in cell volume from leaf to leaf are attributable in the first place to the changing cellular conditions in the apex with the induction of each successive leaf. Sunderland and Brown have demonstrated a decreasing cell

volume in the successive primordia initiated in lupin and this is possibly the case also with sunflower. The smaller average cell volume and the final larger number of cells of the tenth sunflower leaf emphasize that leaf size is not determined by the extent of cell expansion so much as by the extent of the duration of division.

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LITERATURE CITED

Ashby, E., and Wangermann, E. (1950). Studies in the morphogenesis of leaves. V. A note on the origin of differences in cell size among leaves at different levels of insertion on the stem. *New Phytol.* **49**, 189.

Avery, G. S. (1933). Structure and development of the tobacco leaf. *Amer. J. Bot.* **20**, 565.

Brown, R., and Rickless, P. (1949). A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients. *Proc. Roy. Soc.* B. **136**, 110.

von Papen, R. (1935). Beiträge zur kenntnis des Wachstums des Blattspreite. *Bot. Arch.* **37**, 159.

Sunderland, N., and Brown, R. (1956). Distribution of growth in the apical region of the shoot of *Lupinus albus*. *J. Exp. Bot.* **7**, 127.

The Effects of Kinetin, Gibberellic Acid, and Light on Expansion and Cell Division in Leaf Disks of Dwarf Bean (*Phaseolus vulgaris*)

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SUMMARY

The effects of kinetin (Kn), gibberellic acid (G), and light (L) on cell expansion and division in disks from 6-day-old etiolated primary leaves of dwarf bean are described. Cell number was determined by direct counting after disks were digested in a pectinase/EDTA mixture to separate the cells. Kn increased leaf expansion wholly by increasing cell size. G also increased cell size; it increased cell division in the dark but not in the light. Light also increased cell size and cell division; it eliminated the effect of G on cell division but enhanced the effect of G on cell expansion.

INTRODUCTION

It seems well established that kinetin (Kn) makes coleoptile tissue elongate in darkness (Shrank, 1957; Wheeler, 1959) but inhibits elongation of pea-stem tissues both in darkness (Wheeler, 1959) and in light (Brian and Hemming, 1957). Kn makes disks of dwarf-bean leaves expand in darkness, but appears to inhibit growth of disks and intact leaves in light (Humphries, 1958). Miller (1956) showed that Kn and red light had similar effects in causing expansion of dwarf bean leaf disks, and Scott and Liverman (1956) that certain other substituted purines (particularly benzylamino-purine) behaved similarly. Miller (loc. cit.) stated categorically that the expansion results from cell enlargement, and Kuraishi and Okumura (1956), using *Raphanus* leaf disks, demonstrated that disk expansion was a result of cell enlargement and not of division.

Gibberellic acid (G) promotes the growth of all the above tissues both in light and darkness. Our investigation was undertaken to measure the effects and interactions of Kn, G, and light (L) on cell expansion and cell division in leaf disks of dwarf bean.

METHODS

Preparation of leaf disks. Leaf disks (6 mm. diam.) were cut in green light (wavelength 500–570 mμ) from primary leaves of 6-day-old plants grown from graded seeds in darkness at 25° C. Samples of 10 disks were floated for 24 hours on 1 per cent. sucrose solution, alone or containing G or Kn or both at concentrations of 10^{-5} M., with their adaxial surfaces in contact with the

solution. The disks were kept either in darkness or were illuminated by a 'daylight' fluorescent tube producing 270 f.c. at 14 cm. The increase in diameter perpendicular to the chief vein in the disk was measured on an enlarged projected image. Then three disks from each treatment were separately incubated in 1 ml. of pectinase/EDTA mixture (see below) and the cell number per disk was subsequently determined. Each disk was held in boiling water for 10 sec. immediately before incubation to arrest cell division, because the control disks could continue cell division in the enzyme mixture. The number of cells in disks at the end of the treatment was the same whether the disks were boiled or not, except for the control value, indicating that cell division ceased within 24 hours of cutting.

Cell separation. In studies concerned with leaf enlargement a reliable method for determining cell number is essential to distinguish between effects from cell expansion and cell division. The number of cells in a leaf can be determined either by examining sections, or by separating the tissue into individual cells and counting them on a haemocytometer slide. Under properly controlled conditions the latter method is more precise, provided that the macerating agent separates all the cells and does not destroy any. Dilute chromic acid is commonly used but prolonged exposure to it may destroy cells. Pectinase alone (e.g. Chayen, 1952) apparently does not give complete separation, though it may be sufficient for cytological purposes. Ethylene-diamine tetra-acetic acid (EDTA), which has been used to separate cells in some tissues, e.g. mature apple fruit (Letham, 1958), possibly acts by chelating the calcium in the middle lamella. Neither pectinase nor EDTA alone is effective with dwarf-bean leaves, but used together they separate cells completely. The following method was adopted: 100 ml. of citrate buffer (pH 5) was added to 8 g. of a commercial pectinase (Pectinol 100 D, Rohm and Haas), and filtered after standing 15 min. Three parts of enzyme solution were added to 1 part of 0·07 M. EDTA (di-sodium salt), also dissolved in citrate buffer. Although the chelating action of EDTA is greater at a higher pH, it is still appreciable at pH 5, which is also suitable for the action of the enzyme. The mixture must be protected with toluene but excess interferes with counting. Leaf disks were incubated in the mixture (usually 1 or 2 disks per ml.) at 38° C. for 3 days. Small glass phials with tight-fitting, plastic snap-on lids were used to reduce evaporation to a negligible amount, because the calculation of cell number per disk assumes that volume remains unchanged during incubation. At the end of the incubation period the phials were shaken vigorously to break up the disks as much as possible. Cell separation was completed by forcing the suspension several times through the aperture of a fine pipette, which gave clean preparations without clumps of cells. Ten haemocytometer squares from each of two drops were counted in each sample. Although the method worked well for leaves such as bean and tobacco, it is not generally applicable; the cells from leaves with a thick cuticle, such as holly and ivy, did not separate completely and the upper and lower epidermes separated as intact sheets of cells. The pectinase/EDTA

mixture had no effect on leaves of grass or barley but satisfactorily separated root cells of both dwarf and broad bean.

RESULTS

Table I shows the mean diameter of treated dwarf bean leaf disks, measured perpendicular to the chief vein in each disk, the cell numbers per disk determined after maceration and the mean effects and interactions. Kn and G both promote growth in the dark; their effects are significant but their interaction

TABLE I

Mean effects of kinetin (Kn: 10^{-5} M), *gibberellic acid* (G: 10^{-5} M), *and light* (L) *and their interactions on diameter, cell number, and cell size of bean leaf disks*

	Diameter (mm.)	% of mean	Cell no. per disk ($\times 10^6$)	% of mean	Cell size ($mm^2 \times 10^5$)	% of mean
Control	6·0	—	1·28	—	2·85	—
Mean	8·18	—	2·19	—	3·14	—
Kn.	0·12	1·5	−0·11	−5·0	0·31	9·8
G	0·84	10·3	0·10	4·6	0·61	19·4
KnG	−0·02	−0·2	−0·01	−0·4	0·04	1·3
L	2·12	25·9	0·26	11·9	1·28	40·8
LKn	−0·32	−3·9	−0·10	−4·6	−0·16	−5·1
LG	0·32	3·9	−0·10	−4·6	0·34	10·8
LKnG	−0·07	−0·8	0·04	1·8	−0·18	−5·7
L.S.D.	0·08	1·0	0·05	2·3	0·14	4·5

is not, indicating that their effects are additive. The growth-promoting effect of Kn is eliminated by light but that of G is increased. G stimulates cell division in darkness and increases number of cells per disk but has no effect in light. The average effects of Kn in light calculated from the mean effect of Kn and its interaction with light (LKn) given in Table I is $-0{\cdot}11 - 0{\cdot}10 = -0{\cdot}21$ and the effect in darkness is $-0{\cdot}11 + 0{\cdot}10 = -0{\cdot}01$. Thus Kn has no effect on cell division in darkness but inhibits it in light.

The square of disk diameter was divided by the number of cells per disk to give a measure of the surface area per cell, assuming that the number of cells in the transverse direction remained constant (see col. 5, Table I). The relative contributions of cell expansion and cell division to final disk size is more readily appreciated from the effects expressed as a percentage of the mean (Table I). Kn increased leaf expansion wholly by increasing cell size; in the light but not in the dark this effect on cell extension was off-set by decreased cell division. G also increased leaf expansion by increasing cell size, but in contrast with Kn it also increased cell number, although only in the dark. Increased leaf expansion by illumination was caused partly by increased cell division and partly by increase in cell size, but increased cell size was more important than cell division. There is no clear evidence from Table I that light acts by influencing concentrations of Kn and G in the leaf. The only indication of such an effect of light is that illumination eliminated the effect of G on

cell division, but on the contrary it apparently enhanced the effect of G on cell expansion.

DISCUSSION

The results indicate that both L and G increase cell division in isolated portions of bean leaf. Previous evidence also suggested that stems elongate under the influence of G more from cell division than from increase in cell size, cf. Greulach and Haesloop (1958) who found that G did not influence cell size in dwarf-bean internodes. Sachs *et al* (1957, 1959) found that G increased mitotic activity in stem apices and in sub-apical regions of *Hyoscyamus niger* and *Somalus parviflorus*. Bradley and Crane (1957) found that G stimulates cambial activity in apricot stems and Schroeder and Spector (1957) reported that G stimulates callus formation in excised citron mesocarp. Gattridge and Thompson (1958) concluded from measurements of cell and petiole lengths in strawberry that cell division and elongation were both induced by G. Deyson (1959) reported that Kn reduced mitosis and cell elongation in onion roots. Humphries (1958) found that Kn sprayed on to intact bean leaves inhibited growth slightly; this did not occur in the presence of G.

The action of Kn or G evidently depends on external conditions. Kn accelerates disk expansion in the dark but not in the light and depresses cell division in light. Although Kn was first described as a cell-division factor, and under certain circumstances appears to be so, it inhibits growth of leaves and even more of roots. For instance, Kuraishi and Okumura (loc. cit.) observed inhibition in growth of *Brassica campestris* roots and Danckwardt-Lilliestrom (1957) found Kn inhibited growth of isolated roots of *Isatis tinctoria*. De Ropp (1956) reported that elongation induced in sunflower hypocotyl fragments by IAA was suppressed by Kn. Thus there is much evidence that although Kn usually inhibits cell division, under special and as yet undefined conditions it may stimulate division.

In contrast the present experiments indicate that under certain conditions (apparently darkness is one requisite) G accelerates cell division. This is in accord with the observations on stems treated with G. That G depresses cell division in light may explain the conflicting conclusions about the action of G that have been drawn from work with tissue cultures (Nickell, 1958).

LITERATURE CITED

Bradley, M. V., and Crane, J. C. (1957). Gibberellic-stimulated cambial activity in stems of apricot spur shoots. *Science*, **126**, 972.

Brian, P. W., and Hemming, H. G. (1957). Effect of gibberellic acid and kinetin on growth of pea stem sections. *Naturwissenschaften*, **44**, 594.

Chayen, J. (1952). Pectinase technique for isolating plant cells. *Nature*, **170**, 1070.

Danckwardt-Lilliestrom, C. (1957). Kinetin induced shoot formation from isolated roots of *Isatis tinctoria*. *Physiol. Plant.* **10**, 794.

Deyson, G. (1959). Action de la kinétine et de la thiokinétine sur la division et l'elongation cellulaires dans les racines d'*Allium sativium* L. *Compt. Rend.* **248**, 1214.

Gattridge, C. G., and Thompson, P. A. (1958). Effect of gibberellic acid on length and number of epidermal cells in petioles of strawberry. *Nature*, **183**, 197.

Greulach, V. A., and Haesloop, J. G. (1958). The influence of gibberellic acid on cell division and cell elongation in *Phaseolus vulgaris*. *Amer. J. Bot.* **45**, 566.

HUMPHRIES, E. C. (1958). Effect of gibberellic acid and kinetin on growth of the primary leaf of dwarf bean (*Phaseolus vulgaris*). *Nature*, **181**, 1081.

KURAISHI, S., and OKUMURA, F. S. (1956). The effect of kinetin on leaf growth. *Bot. Mag., Tokyo*, **69**, 300.

LETHAM, D. S. (1958). Maceration of plant tissues with Ethylene-diamine-tetra-acetic acid. *Nature*, **181**, 135.

MILLER, C. O. (1956). Similarity of some kinetin and red light effects. *Plant Physiol.* **31**, 318.

NICKELL, L. G. (1958). Gibberellin and growth of plant tissue cultures. *Nature*, **181**, 499.

de ROPP, R. S. (1956). Kinetin and auxin activity. *Plant Physiol.* **31**, 253

SACHS, R. M., and LANG, A. (1957). Effect of gibberellin on cell division in *Hyoscyamus*. *Science*, **125**, 1144.

—— BRETZ, C. F., and LANG, A. (1959). Shoot histogenesis: the early effects of gibberellin upon stem elongation in two rosette plants. *Amer. J. Bot.* **46**, 376.

SCOTT, R. A., and LIVERMAN, J. L. (1956). Promotion of leaf expansion by kinetin and benzylaminopurine. *Plant Physiol.* **31**, 321.

SCHROEDER, C.A., and SPECTOR, C. (1957). Effect of gibberellic acid and indoleacetic acid on growth of excised fruit tissue. *Science*, **126**, 701.

SHRANK, A. R. (1957). Stimulation of *Avena* coleoptile growth by kinetin and some of its analogs. *Plant Physiol.* (*suppl.*), **32**, xlix.

WHEELER, A. W. (1959). *Annual Report Rothamsted Exptl. Stn. for 1958*, p. 77.

High Pressures of Oxygen and Respiration; an Improved Technique and a Demonstration that High Pressures as such may affect the Rate of CO_2 Output from plant Tissues

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SUMMARY

A technique is described which allows the metabolic rate of CO_2 output to be determined during the first 24 hours of treatment with oxygen at high pressures.

With potatoes, and to a lesser extent with apples, the rate of CO_2 output was inhibited by an increase of the absolute pressure to 3 atmospheres, the partial pressure of oxygen remaining at 20 per cent. of 1 atmosphere. The inhibition is attributed to mechanical distortion of the tissue caused by the increase in pressure.

INTRODUCTION

In earlier work on the effect of oxygen at high pressures on apples (Caldwell, 1956) and peas (Turner and Quartley, 1956), CO_2-free air was passed through the container, the rate of CO_2 output being determined on the effluent gas-stream (Turner and Quartley, 1956). In treating with oxygen at a high pressure, a current of CO_2-free oxygen was used and the pressure in the container was allowed to increase without change in the rate of out-flow. With this procedure the CO_2 in the container was diluted initially in proportion to the increase in pressure and this dilution produced a corresponding decrease in the measured rate of CO_2 output; an adjustment period of some 24 hours elapsed before the measured rate of CO_2 output again represented accurately the metabolic rate. Thus the initial influence on the rate of CO_2 production of the change to oxygen at high pressures could not be determined. Moreover, during the adjustment phase the partial pressure of CO_2 in the container increased in proportion to the rise in pressure, which might affect the respiration.

These defects of the earlier technique are illustrated in Fig. 1. After an initial period in which air was passed through the container, a mixture of 7 per cent. oxygen and 93 per cent. nitrogen was substituted, the pressure in the container being increased to 3 atmospheres without change in rate of out-flow of gas. By this procedure the partial pressure of oxygen was held at 21 per cent. of atmosphere, the intention being to avoid any change in rate of respiration due to increased oxygen tension. The immediate large decrease in the observed rate of CO_2 output (Fig. 1, non-compensated samples) is attributed to the initial dilution of the CO_2 in the effluent gas, but after an

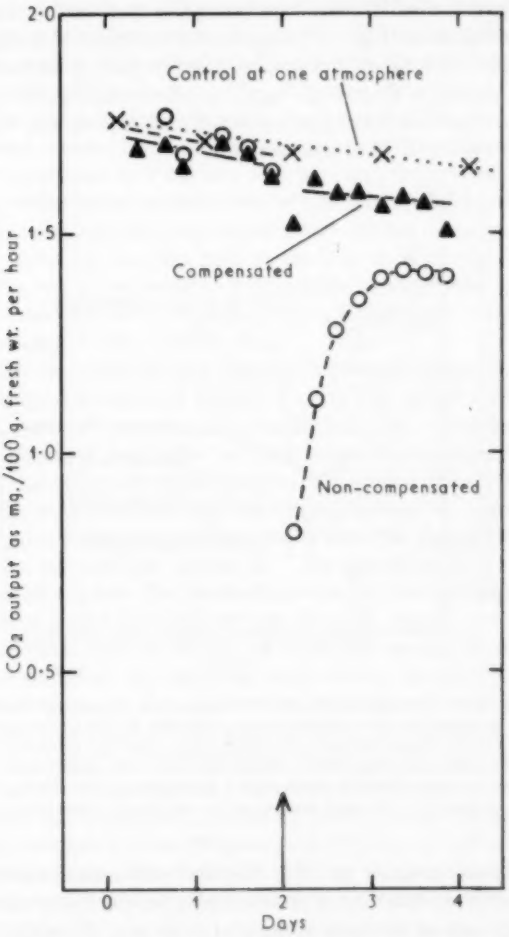

FIG. 1 (January 1957). Changes in the rate of CO_2 output of apples held at 15° C. in air throughout at a pressure of 1 atmosphere; or in a mixture of 7% oxygen and 93% nitrogen at a pressure of 3 atmospheres, the rate of outflow of gas from the container either remaining unchanged (non-compensated samples) or being increased 3-fold (compensated samples). All samples were initially in air at a pressure of 1 atmosphere. The vertical arrow shows the time of increase of pressure. For each treatment the results are mean values of 2 comparable samples.

adjustment phase of 24–30 hours, the measured rate was still below that of the control.

If, however, apples were subjected to a mixture of 7 per cent. oxygen and 93 per cent. nitrogen at a pressure of 3 atmospheres, but the initial dilution of the CO_2 in the effluent gas was compensated by increasing threefold the rate

of outflow of gas, the initial large decrease in the measured rate of CO_2 output was almost eliminated (Fig. 1, compensated samples) although the rate of CO_2 production after 24–30 hrs. was still below that of the control.

Using this technique Pritchard (1959) has shown that there was no decrease of the CO_2 production from green shelled peas during the first 3 hours in oxygen at a pressure of $3\frac{1}{2}$ atmospheres.

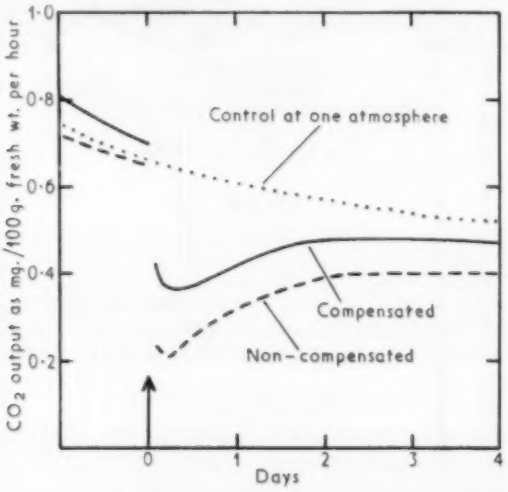

FIG. 2 (October 1956). Changes in the rate of CO_2 output of potatoes held at 15° C. in air throughout at a pressure of 1 atmosphere; or in a mixture of 7% oxygen and 93% nitrogen at a pressure of 3 atmospheres, the rate of outflow of gas from the container either remaining unchanged (non-compensated samples) or being increased threefold (compensated samples). All samples were initially in air at a pressure of 1 atmosphere. The vertical arrow shows the time of increase of pressure. For each treatment the results are mean values of 2 comparable samples.

Effect of partial pressure of CO_2. For the non-compensated samples the CO_2 output after 30 hours at a pressure of 3 atmospheres was some 15 per cent. below the rate of the control samples in air at 1 atmosphere (Fig. 1); for the compensated samples the corresponding decrease was about 5 per cent. For the non-compensated and compensated samples the partial pressures of CO_2 within the containers after 24 hours under high pressure were calculated as being equivalent to about 1·95 and 0·8 per cent. of 1 atmosphere respectively, the latter value being slightly lower than that in air at 1 atmosphere. The larger depression of the rate of CO_2 output after 24–30 hours of the non-compensated samples as compared with the compensated samples may thus be due to the higher partial pressure of CO_2 and possibly of other volatile compounds. A similar difference is shown for potatoes in Fig. 2.

Influence of high pressures on respiration. Caldwell (1956) concluded that inhibition of the respiration of apples subjected to high pressure was caused

by the high partial pressure of oxygen and not by high pressures as such. Though confirming this conclusion in general, our results indicate that with potatoes, and to a lesser extent with apples, some inhibition of respiration was due simply to the high pressure.

The technique used to obtain the data for potatoes was essentially similar to that described above. With the non-compensated samples the transition from air at 1 atmosphere to a mixture of 7 per cent. oxygen and 93 per cent. nitrogen at a pressure of 3 atmospheres produced the expected large initial decrease in measured rate of CO_2 output which settled down to a rate which was below the value for samples held in air, Fig. 2. With the samples compensated for the rise in pressure to 3 atmospheres there was also an initial, though smaller, decrease in the measured rate of CO_2 output; subsequently the rate increased nearly to the control value.

The results for the compensated samples of potatoes thus differ from those for the compensated samples of apples, Fig. 1. The initial marked decrease in rate of CO_2 output from the compensated samples of potatoes appears to be due to an effect on respiration of high pressures as such. One possible explanation of this effect is that the tissues are distorted during the increase in pressure, though usually mechanical distortion increases the rate of respiration (Audus, 1935). An alternative explanation is that the distortion reduces the size either of the intercellular spaces or of the apertures in the skin through which oxygen diffuses into the intercellular spaces; the consequent decrease in oxygen tension might diminish the rate of respiration.

It seemed possible that the degree of distortion would be reduced if the pressure in the container was increased more slowly. In a further experiment with potatoes the pressure was increased at the usual rate with 2 samples and more slowly with 2 samples. There was, however, no difference in the results, though again the respiration of the compensated samples was inhibited.

The above results suggest that the influence of high pressures of oxygen on respiration may be compound, consisting partly of an effect of increased oxygen tension and partly of an influence of distortion. If, however, the inhibition of respiration caused by distortion was due to a decrease in the cellular content of oxygen, this inhibition would not be expected to occur in oxygen at high pressures.[1]

ACKNOWLEDGEMENTS

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I wish to thank Professor G. E. Briggs, F.R.S., and Dr. L. W. Mapson for advice and Miss E. L. Presley who conducted the experimental work.

[1] There appears to be no effect of pressure as such with green peas during the first 3 hours in high oxygen (see above, p. 88).

LITERATURE CITED

AUDUS, L. J. (1935). Mechanical stimulation and respiration in the Cherry-Laurel. *New Phytol.* **34**, 386.

CALDWELL, J. (1956). Studies in the respiration of apples at various pressures of oxygen. *J. Exp. Bot.* **7**, 326.

PRITCHARD, G. G. (1959). Ph.D. Thesis, Cambridge University.

TURNER, E. R., and QUARTLEY, C. E. (1956). An inhibition of respiration in peas induced by oxygen poisoning. *J. Exp. Bot.* **7**, 362.

Inhibition of Nitrogen Fixation in Non-Legume Root Nodules by Hydrogen and Carbon Monoxide

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SUMMARY

The effect of the presence of hydrogen and of carbon monoxide on the fixation of nitrogen in detached root nodules of non-legumes has been studied, fixation being measured by the use of ^{15}N. Parallel tests on legumes (pea and soya bean) have been included. Fixation in the nodules of *Casuarina*, *Alnus*, and *Myrica* is inhibited in the presence of substantial proportions of hydrogen, to a degree resembling that shown in legumes. Fixation in *Alnus* and *Myrica* is arrested in the presence of small proportions of carbon monoxide, and here again the sensitiveness is of the same order as in legumes.

INTRODUCTION

It is well known that in those instances of biological fixation of nitrogen which have been more extensively studied the process is inhibited in the presence of suitable partial pressures of hydrogen or of carbon monoxide. Hydrogen-inhibition was first detected in legume nodules. In long-term experiments utilizing clover plants wholly enclosed in large bottles and exposed to various gas mixtures, Wilson and his collaborators (see Wilson, 1940) showed that fixation was about 75 per cent. inhibited in the presence of 0·6 atm. hydrogen, with gaseous nitrogen supplied at 0·2 atm. partial pressure. Hydrogen-inhibition in *Azotobacter* was demonstrated by Wyss and Wilson (1941) and with less certainty for *Nostoc* by Burris and Wilson (1946), a smaller inhibitory effect being detected in *Clostridium* by Rosenblum and Wilson (1950). Lind and Wilson (1941), following some earlier indications, showed that fixation in nodulated clover plants was almost suppressed in the presence of as little as 0·0005 atm. carbon monoxide. Lind and Wilson (1942) and Wilson and Lind (1943) found that with *Azotobacter* a partial pressure of about 0·005 atm. of carbon monoxide is necessary for the complete inhibition of fixation, a similar conclusion being reached by Virtanen (1953) in respect of *Clostridium*. Fixation in *Nostoc* is also sensitive to carbon monoxide (Burris and Wilson, 1946).

In the work now to be described the sensitiveness towards hydrogen and carbon monoxide of the nitrogen fixation proceeding in the root nodules of certain non-legume Angiospermous plants has been examined. The general features of this fixation have been reviewed by Bond (1958).

METHODS

Observations have been made in respect of the nodule-bearing non-legumes *Alnus glutinosa*, *Casuarina cunninghamiana*, and *Myrica gale*. In order to secure comparable data for legume nodules, parallel experiments were made with pea (*Pisum sativum*) and soya bean (*Glycine max*). Nodulated plants of all these species were grown in water culture (Crone's solution, free of combined nitrogen) in the greenhouse, cultural details for the non-legumes having been described previously (Bond, 1951; Ferguson and Bond, 1953; Bond, 1957*a*). The rhizobia employed for legume inoculation were Professor Virtanen's HX strain for pea and Wisconsin 505 for soya bean.

With the non-legumes, nodules were detached and then quickly exposed to atmospheres which included nitrogen labelled with ^{15}N. For a limited period fixation continues relatively strongly in these non-legume nodules after detachment and is readily measured by the isotopic method even though the whole of the nodule nitrogen is assayed (Bond, 1957*b*). The survival of fixation in detached legume nodules is less satisfactory, and in view of this in the present studies short lengths (3 to 5 cm.) of abundantly nodulated roots of the legumes were utilized, in the belief (verified below) that fixation would be considerably enhanced as compared with completely detached nodules.

Gaseous nitrogen containing 34 atom per cent. excess ^{15}N was prepared by allowing sodium hypobromite to react with labelled ammonium nitrate over mercury in a modified nitrometer. Gaseous oxygen was obtained from a cylinder or by the action of washed yeast on hydrogen peroxide. Hydrogen and argon were drawn from cylinders, and carbon monoxide prepared by reaction between formic and sulphuric acids. Suitable mixtures of these gases were prepared, and it will be convenient to present the composition of the mixtures on a volume percentage basis. Since the mixtures were prepared and utilized at a pressure of 1 atm., conversion to a partial pressure basis is of course effected by division by 100. In all cases 10 per cent. of nitrogen and 20 per cent. of oxygen were present, together with various proportions of hydrogen or carbon monoxide, argon (an inert gas so far as fixation is concerned) being used to bring the total pressure to 1 atm.

Samples of 1 gm. fresh weight of detached nodules, or of nodulated roots in the case of the legumes, were placed along with 1 ml. of Crone's solution in stout glass specimen tubes (capacity 28 ml.) fitted with stopcocks, the tubes being then attached to a manifold and evacuated. The appropriate gas mixture was then admitted to the tubes from a gas storage bottle also attached to the manifold, water being subsequently run into the storage bottle to drive the gas into the sample tubes to a pressure of 1 atm.

The charged tubes were incubated at 23° C. for periods to be indicated later. The non-legume samples were then submitted to the Kjeldahl process, the distillates after titration and reacidification being evaporated to a small volume, part of which was subject to mass spectrometric assay. The mean total nitrogen contents of the 1 gm. samples utilized in the experiments were

as follows (mg.): *Myrica* 6·0, *Alnus* 4·9, *Casuarina* 3·4, pea 2·8. In some instances the legume samples were extracted with 3N HCl and this acid-soluble nitrogen fraction alone assayed, following the procedure adopted by Magee and Burris (1954) for completely detached nodules. The total nitrogen in the acid extracts from three 1 gm. samples bulked together was approximately 2 mg. for pea and 3 mg. for soya bean. Data to be presented below indicate that when nodulated root portions of legumes are employed this extra step of acid extraction is actually unnecessary.

RESULTS

TABLE I

*Effect of hydrogen on fixation in various root nodules**

Nodule Material	Hydrogen supplied in gas mixture	Mean atom per cent. excess ^{15}N in samples after exposure	Per cent. inhibition of fixation by hydrogen
Pea	None	0·301 (0·277–0·322)	—
	60 per cent.	0·068 (0·043–0·098)	77
Soya bean	None	0·860	—
	60 per cent.	0·125	86
Casuarina	None	0·544 (0·523–0·556)	—
	60 per cent.	0·089 (0·062–0·104)	84
Alnus	None	0·030 (0·024–0·034)	—
	60 per cent.	0·004 (0·001–0·008)	87
Myrica	None	0·128 (0·069–0·198)	—
	60 per cent.	0·015 (0·009–0·020)	88

* The experiments were carried out in July and August 1958 with triplicate samples at each treatment, the samples being exposed to the gas mixtures for 18 hours. The ^{15}N data are based on the assay of the whole of the nitrogen of the samples except in the case of soya bean, where acid-soluble nitrogen alone from bulked samples was assayed. The figures in parentheses show the maximum variation in ^{15}N content between comparable samples.

Hydrogen-inhibition. In Table I are assembled data indicating the effect on the fixation of nitrogen (a relative measure of the latter being provided by the increase in combined ^{15}N content shown by the samples) of the presence of 60 per cent. of hydrogen, replacing that proportion of argon in the atmosphere supplied. The results with pea and soya bean confirm that the hydrogen-inhibition of fixation in legume nodules detected by previous workers using a quite different technique was reproduced under the present author's conditions. The high level of enrichment and the good replication shown by the pea material in the absence of hydrogen indicate that the use of nodulated root portions in legume studies has much to recommend it. In the three non-legumes a strong hydrogen-inhibition is revealed and is approximately of the same order of intensity as in the legumes. The excellent level of fixation and replication displayed by the *Casuarina* samples in the absence of hydrogen underlines the undoubted special suitability of these particular nodules for experimental study. Fixation was lower in *Alnus* and *Myrica* and replication poorer in the latter, but the hydrogen effect is still perfectly clear.

Table II presents the results of a further experiment with *Alnus* in which an intermediate level (20 per cent.) of hydrogen was included and was found to produce a substantial degree of inhibition of fixation.

TABLE II

Effect of proportion of hydrogen present on fixation in Alnus *nodules**

Hydrogen supplied in gas mixture	Atom per cent. excess ^{15}N in samples after exposure	Per cent. inhibition of fixation by hydrogen
None	0·074, 0·067, 0·051 — Mean = 0·064	—
20 per cent.	0·036, 0·014, 0·044 — Mean = 0·031	52
60 per cent.	0·017, 0·005, 0·016 — Mean = 0·013	80

* The triplicate samples at each treatment were exposed to the gas mixtures for 5 hours.

TABLE III

*Effect of carbon monoxide on fixation in various root nodules**

Nodule Material	Per cent. CO initially present in gas mixture	Mean atom per cent. excess ^{15}N in samples after exposure	Per cent. inhibition of fixation by CO
Pea	0	1·523	—
	0·01	1·474	3
	0·1	0·318	79
	1·0	0·016	99
Myrica	0	0·165	—
	0·01	0·148	10
	0·1	0·057	66
	1·0	0·004	98
Alnus	0	0·058	—
	0·01	0·092	−59
	0·1	0·040	31
	1·0	0·001	98

* The experiments were carried out in August 1958, the pea samples being exposed to the gas mixtures for 18 hours and those of the non-legumes for 5 hours. Triplicate samples of pea and *Myrica* material were included for each treatment, but only duplicate samples of *Alnus*. ^{15}N assays were carried out on the acid-soluble fraction of the pea material but on the whole of the nitrogen in the non-legume material.

Carbon monoxide-inhibition. In view of the reasonably satisfactory agreement between replicates shown in the above experiments, in those now to be described comparable samples were combined prior to ^{15}N assay. Table III provides data illustrating the effect of increasing levels of carbon monoxide on fixation in nodules of pea and of two non-legumes. In pea there is continuous depression of fixation as the proportion of carbon monoxide is

increased, but it will be noted that the level of this gas necessary for fixation to be virtually completely suppressed is apparently much higher than in the experience of previous workers using a different technique (p. 91). This draws attention to a disadvantage of the isotopic technique, namely that with the present cost of ^{15}N it is scarcely possible to employ a large volume of labelled gas relative to the size of the nodule sample. In the present tests, at the lower levels of carbon monoxide the absolute amount of that gas in contact with a sample was exceedingly minute and was doubtless insufficient to affect the enzymic or other catalytic centres to the extent that would obtain given a larger volume of a similar gas mixture. For this reason the data in Table III are not directly comparable with those obtained by other techniques.

Of the non-legume material, *Myrica* nodules show a close resemblance to those of pea in their reaction to increasing level of carbon monoxide. With *Alnus* nodules there was apparently a stimulatory effect on fixation on the part of the lowest level of carbon monoxide, but although there are previous records of such an effect in respect of other cell processes (see Smith, 1949), in the present instance variation in initial fixatory activity between samples may have been responsible, particularly since two samples only of *Alnus* nodules were included for each treatment. With all three nodular types the initial level of carbon monoxide necessary for fixation to be virtually suppressed lies between 0·1 and 1·0 per cent. under the conditions of the experiments.

DISCUSSION

The nitrogen-fixing mechanism in non-legume root nodules is shown by data advanced above to resemble that in other biological material in that the presence of a substantial partial pressure of hydrogen results in a derangement leading eventually to a cessation of fixation. A further feature demonstrated by Wilson, Lee, and Wyss (1941) for clover and by Wyss, Lind, Wilson, and Wilson (1941) for *Azotobacter* was that this hydrogen-inhibition of fixation appeared to involve a competition between nitrogen and hydrogen, since the inhibition occasioned by a given partial pressure of hydrogen could be diminished by increasing the partial pressure of nitrogen. This aspect has not yet been investigated for the non-legume nodules. Although the theoretical interest of the hydrogen-inhibition of biological fixation of nitrogen has long been appreciated, the present understanding of the mechanism of fixation does not permit of the certain identification of the particular stage in the process at which the inhibition operates; the matter has been reviewed recently by Wilson (1958).

The experiments involving carbon monoxide indicate that the sensitiveness to this gas in the particular non-legume nodules examined is of the same order as in legume nodules. This is a finding of some interest. Various authors (e.g. Keilin and Smith, 1947) have surmised that the special sensitivity towards carbon monoxide of the fixation in legume nodules as compared with that in other material such as *Azotobacter* might be due to an inactivation of the nodule haemoglobin. The examination of non-legume nodules (some of which

are frequently red in colour) for the presence of haemoglobin has yielded negative results (Smith, quoted by Bond, 1951; Egle and Munding, 1951), but these authors reported that other haemin compounds were unusually abundant in the nodules. There is the obvious possibility that these compounds play some catalytic role in the fixation and that they, like haemoglobin, are inactivated by very low concentrations of carbon monoxide.

The findings reported in this paper extend the list of respects in which similarity between fixation of nitrogen in non-legume nodules and in other material has been established. In addition to the physiological similarities now demonstrated it has been shown that there are certain resemblances in respect of the biochemistry of the fixation (Leaf, Gardner, and Bond, 1958; 1959).

The author is greatly indebted to Dr. R. H. Burris for his kindness in carrying out the ^{15}N assays reported in this paper. He also acknowledges the efficient technical assistance given by Miss K. McCormick.

LITERATURE CITED

Bond, G. (1951). The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. *Ann. Bot.* N.S. **15**, 447–59.

—— (1957*a*). The development and significance of the root nodules of *Casuarina*. Ibid. **21**, 373–80.

—— (1957*b*). Isotopic studies of nitrogen fixation in non-legume root nodules. Ibid. 513–21.

—— (1958). Symbiotic nitrogen fixation by non-legumes. In *Nutrition of the Legumes*. Butterworths, London.

Burris, R. H., and Wilson, P. W. (1946). Characteristics of the nitrogen-fixing enzyme system in *Nostoc muscorum*. *Bot. Gaz.* **108**, 254–62.

Egle, K., and Munding, H. (1951). Über den Gehalt an Häminkörpern in den Wurzelknöllchen von Nicht-Leguminosen. *Naturwissenschaften*, **38**, 548–9.

Ferguson, T. P., and Bond, G. (1953). Observations on the formation and function of the root nodules of *Alnus glutinosa* (L.) Gaertn. *Ann. Bot.* N.S. **17**, 175–88.

Keilin, D., and Smith, J. D. (1947). Haemoglobin and nitrogen fixation in root nodules of leguminous plants. *Nature*, Lond. **159**, 692.

Leaf, G., Gardner, I. C., and Bond, G. (1958). Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Alnus*. *J. Exp. Bot.* **9**, 320–31.

—— —— —— (1959). Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Myrica*. *Biochem. J.* (in the press).

Lind, C. J., and Wilson, P. W. (1941). Mechanism of biological nitrogen fixation. VIII. Carbon monoxide as an inhibitor for nitrogen fixation by red clover. *J. Amer. Chem. Soc.* **63**, 3511–4.

—— —— (1942). Carbon monoxide inhibition of nitrogen fixation by *Azotobacter*. *Arch. Biochem.* **1**, 59–72.

Magee, W. E., and Burris, R. H. (1954). Fixation of N_2^{15} by excised nodules. *Plant Physiol.* **29**, 199–200.

Rosenblum, E. D., and Wilson, P. W. (1950). Molecular hydrogen and nitrogen fixation by *Clostridium*. *J. Bact.* **59**, 83–91.

Smith, J. D. (1949). Haemoglobin and the oxygen uptake of leguminous root nodules. *Biochem. J.* **44**, 591–8.

Virtanen, A. I. (1953). Assimilation of molecular and combined nitrogen by micro-organisms. In Symposium on Microbial Metabolism. Ist. Superiore di Sanita. Rome.

Wilson, P. W. (1940). *The Biochemistry of Symbiotic Nitrogen Fixation*. University of Wisconsin Press, Madison.

—— (1958). Asymbiotic nitrogen fixation. In *Encyclopedia of Plant Physiology*, VIII (ed. W. Ruhland), Springer-Verlag, Berlin.

WILSON, P. W., LEE, S. B., and WYSS, O. (1941). Mechanism of symbiotic nitrogen fixation. V. Nature of inhibition by hydrogen. *J. biol. Chem.* **139**, 81–101.

—— and LIND, C. J. (1943). Carbon monoxide inhibition of *Azotobacter* in microrespiration experiments. *J. Bact.* **45**, 219–32.

WYSS, O., LIND, C. J., WILSON, J. B., and WILSON, P. W. (1941). Mechanism of biological nitrogen fixation. VII. Molecular H_2 and the pH_2 function of *Azotobacter*. *Biochem. J.* **35**, 845–54.

—— and WILSON, P. W. (1941). Mechanism of biological nitrogen fixation. VI. Inhibition of *Azotobacter* by hydrogen. *Proc. Nat. Acad. Sci. U.S.A.* **27**, 162–8.

Phosphorus Compounds, Nuclease, and Phosphatase Activities in Healthy and Tumorous Stem Tissues of *Datura stramonium* L.

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SUMMARY

The phosphorus compounds, nuclease, and phosphatase activities in healthy and tumorous stem tissues of *Datura* were compared.

The very high level of P in the tumours comes from increased amounts of acid-soluble phosphorus, lipid-phosphorus, ribonucleic acid phosphorus, and deoxyribonucleic acid phosphorus.

The activities of ribonuclease, deoxyribonuclease, and glycerophosphatase were all considerably higher in the tumours than in the stem tissue.

INTRODUCTION

THERE is an extensive and now classical literature on the crown gall as experimental material in the study of plant tumours and both E. F. Smith and Jensen (cit. by Braun, 1954) emphasize similarities between crown gall and animal cancer. Recently, attention was directed by Brody and Ballis (1958) to the different patterns of the ribonuclease and deoxyribonuclease activities in normal and neoplastic growth. They found that the content of RNA and DNA was greater in many human malignant tumours than in the normal tissue. The ribonuclease activity in some tumours was the same as in the tissue of origin, in others it was lower. The deoxyribonuclease activity of the neoplastic tissues studied did not differ greatly from those of the tissue of origin. The only exception was a hepatic carcinoma which had a deoxyribonuclease activity of only 25–30 per cent. that of healthy liver tissue.

The present paper is concerned with the determination of the phosphorus compounds in healthy and tumorous *Datura* stem tissues. The enzymic activities concerned with the breakdown of some of the phosphorus compounds have also been studied.

The total phosphorus content of plant tumours has been reported by a number of workers (Klein, 1952; Lee, 1952; Manigault and Jussier [cited by Braun, 1954]; Neish and Hibbert, 1940; Stapp and Pfeil, 1939) to be considerably higher than in normal tissue. Phosphorus supplied as P^{32} was found by Tsao and Whaley (1950) to be accumulated more extensively in tumour than in normal tissue of *Bryophyllum*. Klein (1952) reported increases in phosphorus compounds in tomato tumour tissue as early as 5 days after

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inoculation. The increased concentration was found to be due to increased amounts of orthophosphate and acid-soluble organic phosphates. Within 14 days after inoculation the total phosphorus content was twice that of the control and over half of this increase was due to an increase in the acid-soluble organic fraction. During later stages of tumour development the relative concentration of the acid-soluble organic fraction decreased during development until this fraction formed about the same proportion of the total phosphorus as in the control.

MATERIAL AND METHODS

Plants of *Datura stramonium* L. about 8 cm. high were inoculated by needle with *Agrobacterium tumefaciens* and used 6–8 weeks after inoculation. Sections of stem tissue were taken from these plants and also from tissues of the crown gall tumours resulting from infection with the bacterium. Similar stem sections taken from healthy plants of the same age were used as controls.

Phosphorus compounds of stems and crown gall tumours were fractionated into four groups by the method described by Holden (1952):

1. *P soluble in cold 0·2 N. perchloric acid.* 6 g. samples of stems and 3 g. of tumours were ground in a porcelain mortar with 20 ml. 0·2 N. $HClO_4$. The acid was not left in contact with the tissue longer than 10 min. After removal of the extract by centrifuging the tissue was washed with 10 ml. of 0·2 N. $HClO_4$ and the extracts combined. The total P and orthophosphate of this fraction were determined. The bound P was calculated from the difference between the total and inorganic P.

2. *P soluble in ethanol-ether.* 3 successive portions: 20, 10, 10 ml. of cold, neutral ethanol-ether (3:1) were sufficient to remove all colour and the removal of P paralleled the removal of pigments.

3. *P soluble in N. $HClO_4$ at room temperature.* RNAP fraction was extracted by soaking the fibre in 20 ml. N. $HClO_4$ overnight, i.e. about 16–18 hours at room temperature. After removal of the extract the tissue was washed with 10 ml. N. $HClO_4$ and the N. $HClO_4$ extracts pooled.

4. *P soluble in N. $HClO_4$ at 37° C.* DNAP fraction was extracted by suspending the fibre in 20 ml. N. $HClO_4$ and incubating at 37° C. overnight. After removal of the extract the tissue was washed with 10 ml. N. $HClO_4$ and the extracts pooled.

Phosphorus was determined colorimetrically by a modification of the method of Kuttner and Lichtenstein described by Holden and Pirie (1955*b*). Values for inorganic P were obtained by developing the colour in samples without incineration.

Enzyme activities. Extracts of crown gall tumours and normal tissues of the stems of *Datura* were used for the determination of the activities of the enzymes specified. 2 g. of crown gall tumours and stem tissue were ground with 5 ml. of a suitable buffer solution in a porcelain mortar and the extract squeezed out by hand through strong cotton cloth. The suspension was centrifuged at 1,500 g. for 5 minutes and the green deposit removed. The clear

supernatant fluid was used as enzyme solution. Activities were measured by the methods described by Holden and Pirie (1955*a*; 1955*b*).

Ribonuclease. Commercial yeast nucleic acid purified by dialysis in a cellophane sac against distilled water which was changed frequently for several days, at 0° C., was used as the substrate. The liberation of P not precipitable by uranyl nitrate in trichloroacetic acid from yeast nucleic acid was used to measure ribonuclease activity.

For the tests a total volume of 4 ml. containing 400 mg. P per litre was incubated in 0·25 M. citrate buffer, pH 6 at 37° C. with 0·3 ml. of enzyme solution. 0·5 ml. samples were removed at intervals of 0·5, 1, and 2 hours, diluted with an equal volume of water and precipitated by adding 1 ml. of uranyl nitrate solution 0·5 per cent. (w/v) in 2·5 per cent. (w/v) TCA. After standing the tubes on ice for 30 minutes the precipitates were centrifuged down (at 2,400 g. for 5 minutes) and total P determined in the supernatants.

Deoxyribonuclease. Activity was determined similarly, using the sodium salt of deoxyribonucleic acid from thymus in a volume of 4 ml. containing 0·4 g./l. P, sodium diethylbarbiturate + sodium acetate buffer 14·3 mM. (pH 6) and enzyme solution. 1 ml. of stem extract or 0·5 ml. of tumour extract was used.

Samples were removed at intervals, diluted with an equal volume of water, and precipitated by adding an equal volume of 0·5 N. HCl. The precipitates were centrifuged down and P was determined in the supernatant fluid.

Phosphomonoesterase. The amount of inorganic P liberated from sodium glycerophosphate was used as a measure of enzyme activity. A volume of 4 ml. containing glycerophosphate to give 0·6 g./l. P was incubated in 0·25 M. citrate buffer (pH 5) at 37° C. with 0·3 ml. of enzyme solution.

The samples removed at intervals were pipetted directly into the mixture of H_2SO_4 and molybdate, and the colour was developed with $SnCl_2$ as in the method for total P.

Dry matter. Samples were dried in an oven at 105° C. overnight.

pH measurements were made with a glass electrode.

RESULTS

Table I shows the results of a typical experiment on the fractionation of phosphorus in fresh tissue of healthy stems, stems inoculated with *Agrobacterium tumefaciens* and tumours from the same stems.

The total phosphorus per g. of dry matter in the stems inoculated with *Agrobacterium tumefaciens* was about 30 per cent. lower than in healthy stems. The various P fractions as a percentage of the total P were about the same in both tissues. In the tumours the total phosphorus per g. of dry matter was significantly higher than in the stems. The highest increase (10–14 times) was noticed in the acid-soluble P fraction and it formed a much higher percentage of the total P in tumour tissue than it did in stem tissue. This increase was due mainly to an increased amount of inorganic P but P in bound form also increased 4–5 fold. The phospholipid P per g. of dry matter was 3–4 times

higher in tumour tissue, but as a percentage of the total P was about half that of the same fraction in stem tissue. Similarly, RNAP was 3–4 times higher in the tumours than in stem tissue, but formed a much lower percentage of the total P. DNAP per g. of dry matter was 5–6 times higher, but as a percentage of total P was about the same in the tumours as in the healthy stems and slightly lower than in inoculated plants.

TABLE I

Fractionation of phosphorus in 6 g. of fresh tissue of stems and 3 g. of tumours (known dry matter contents) extracted successively as shown in the table

		P in healthy stems		P in stems inoculated with *A. tumefaciens*		P in tumours	
	Extractant	mg./g. of dry matter	% of total	mg./g. of dry matter	% of total	mg./g. of dry matter	% of total
1.	0·2 N. $HClO_4$ 30 ml.						
	acid-soluble bound P	0·20	34·7	0·16	34·9	0·82	64·5
	orthophosphate	0·31		0·21		4·52	
2.	Ethanol-ether (3:1), 40 ml. i.e.						
	lipid P	0·54	34·9	0·37	34·9	1·44	17·4
3.	N. $HClO_4$ R.T. overnight 30 ml. i.e.						
	RNAP	0·31	21·0	0·23	21·7	0·90	10·8
4.	N. $HClO_4$ 37° C. overnight 30 ml. i.e.						
	DNAP	0·11	7·4	0·09	8·5	0·59	7·3
	Total	1·47	—	1·06	—	8·27	—

Because of high levels of P compounds found to accumulate in the tumour tissue it seemed of some interest to investigate possible changes in the levels of activity of certain enzymes concerned with phosphorus metabolism. The choice of enzymes examined was affected by the recent investigations of Brody and Ballis (1958) on the nucleic-acid content and nuclease activities of a number of human and animal malignant tumours and Klein's supposition that the remarkable increase in the components of the acid-soluble P fraction could be in connexion with the glycolytic activity of the tumours.

Table II shows the results of a typical experiment on measurement of enzyme activities of RNase, DNase, and GPase in healthy and tumorous stem tissues. The enzyme activity is expressed as units per g. fresh tissue according to the definition of an enzyme unit by Holden and Pirie (1955*a*), viz. the amount of enzyme needed to make in 1 hour 31 mg. P per litre, soluble in the nucleic acid precipitant, or to increase the amount of phosphate-P by 31 mg. per litre.

The activity of RNase and GPase per g. wet weight of the stem tissue was approximately the same in both healthy and inoculated plants. The activity

of DNase was, however, always rather higher in the stems inoculated with *A. tumefaciens* than in healthy stems; in the experiment given (Table II) it was increased by 50 per cent. The activity of the three enzymes of the tumour tissue was higher (1·5–3 times) than in healthy and inoculated stems.

TABLE II

Enzyme activities in units/g. fresh weight of healthy and tumorous stem tissues

Enzyme	Stems		Tumours
	Healthy	Inoculated with *Agrobacterium tumefaciens*	
RNase	3·9	3·3	5·9
DNase	0·62	0·89	2·1
GPase	4·6	4·6	8·1

DISCUSSION

The very high phosphorus level (characteristic of the metabolism of cancer) in the tumours of *Datura* comes from increased amounts of all the various fractions of P compounds, and there seem to be some similarities in the accumulation of phosphorus compounds in plant and animal malignant tumours.

The remarkable increase in the P compounds of the acid-soluble fraction of plant tumours was found by Klein (1952) to be due to increased amounts of orthophosphate and acid-soluble organic phosphates. Waterhouse, Terepka, and Sherman (1952) showed an apparently high concentration of P as an electrolyte in certain human malignant tissues. The increase of the phospholipid fraction in the tumours of *Datura* seems to confirm some findings on animal material. Biesele and Goldhaber (1955) noticed a massive accumulation of lipid droplets in the cytoplasm of malignant tissue of mouse fibroblast and of Sarcoma 180 cells. Bloor and Haven (1955) found a significantly higher phospholipid content in the intestine of rats bearing Walker carcinoma 256 as compared with that of normal rats.

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LITERATURE CITED

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Transamination and the Oxidation of Some Amino-acids by Mung Bean Mitochondria

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SUMMARY

The transaminases for the reaction of α-ketoglutarate with the amino-acids L-alanine, L-aspartic acid, glycine, L-leucine, L-ornithine, and L-valine have been shown to be associated with mung bean mitochondria. Aspartate-glutamate and alanine-glutamate transaminases are two different enzymes both of which are reversible and stimulated by pyridoxal-5′-phosphate.

Aspartate is deaminated, with associated oxygen uptake, by mitochondria from 1-day seedlings, but there is no oxygen uptake with 2-day mitochondria and aspartate, nor with 1- or 2-day mitochondria and L-alanine, glycine, L-leucine, or L-valine although these amino-acids are all deaminated. The change in aspartate metabolism with increased age of the seedlings is probably due to the reorganization of the enzyme systems which resulted in DPNH being largely oxidized by malic dehydrogenase and oxaloacetate, instead of mainly by a DPNH oxidase.

INTRODUCTION

ENZYMIC transamination was first demonstrated by Braunstein and Kritzmann (1937) in muscle preparations. Transamination was later shown to occur in pea plants by Virtanen and Laine (1938) and now has been described in a variety of higher plants (Albaum and Cohen, 1943; Leonard and Burris, 1947; and Rautanen, 1946). Wilson *et al.* (1954), with the use of radioactive techniques, showed that seventeen naturally occurring amino-acids could participate in transaminase reactions catalysed by plant extracts and particulate preparations from lupins.

Braunstein and Bychkov (1939) studied the deamination of amino-acids by kidney homogenates and suggested that the reaction involved the coupling of transaminases and glutamic dehydrogenase. As glutamic dehydrogenase is present in higher plants (Damodaran and Nair, 1938) and has been shown to be associated with mitochondria in peas (Davies, 1956) and in oats (Rautanen and Tager, 1955), the coupling of transaminase and glutamic dehydrogenase may occur in plants also and be the mechanism for oxidative deamination and, in reverse, a synthetic pathway for certain amino-acids. The present paper is concerned with the ability of mung bean mitochondria to catalyse the transamination and oxidation of some amino-acids.

METHODS

The growth of mung bean seedlings and the isolation of mitochondria from them have been described in a previous paper (Bone, 1959). Glutamic acid and aspartic acid were estimated using lyophilized *Clostridium welchii* (Meister

et al., 1951) and malate-adapted *Lactobacillus arabinosus* was used to estimate malic acid (Nossal, 1952). The production of oxaloacetic acid by aspartate-glutamate transaminase reaction was measured by decarboxylation with aluminium sulphate (Krebs and Eggleston, 1945). Ammonia was distilled in Conway units and then estimated by a nesslerization procedure. Conventional Warburg manometric techniques were used and carbon dioxide was measured by the direct method. Reactions involving diphosphopyridine nucleotide (DPN) were followed by measuring the changes in optical density at 340 mμ with a Unicam spectrophotometer, model SP. 500. Spectrophotometric experiments were carried out at room temperature (20° C.); all other experimental systems were incubated at 30° C.

RESULTS

Transaminases

Enzymes capable of catalysing the transfer of the amino group from several amino-acids to α-ketoglutaric acid were found to be associated with the bean mitochondria. Enzyme activities for the amino-acids L-alanine, L-aspartate, glycine, L-leucine, and L-ornithine utilizing α-ketoglutaric acid as the amino group acceptor were assayed by measuring the glutamate formed (Table I).

TABLE I

Transaminases of mitochondria

Amino group donors	Glutamate formed in 1 hour (μl. carbon dioxide)	
	1-day seedlings	2-day seedlings
L-aspartate	162	187
L-alanine	43	107
L-leucine	0	17
L-ornithine	—	21
Glycine	—	21

The reaction system contained: 40 μmol. amino-acid, 40 μmol. potassium α-ketoglutarate, 100 μmol. phosphate buffer, pH 7·4 and 0·5 ml. mitochondrial suspension. Total volume 2·0 ml. Reaction stopped by the addition of 0·3 ml. of 2N HCl.

The transaminase activities of mitochondria were found to vary with the age of the seedlings from which the particles were isolated and in the case of alanine-glutamate and leucine-glutamate transaminases marked differences in activity occurred (Table I). Aspartate-glutamate transaminase was found to be very active and the activity of mitochondria isolated from either 1- or 2-day-old seedlings was fairly constant. This is illustrated by the progress curves of the reaction when using the two types of mitochondria (see Fig. 1); the hyperbolic character of the curves is due to an equilibrium position that is in favour of the reactants ($K \simeq 0{\cdot}1$). As these mitochondrial preparations from 1- and 2-day-old seedlings contained small amounts of the acids,

pyruvate, and α-ketoglutarate (0·1 μmol. of each acid in 0·5 ml. of mitochondrial suspension) slight transamination occurred in the control systems.

Coenzyme requirement. The effect of pyridoxal phosphate (20 μg. of the free acid/ml. of reaction mixture) on the mitochondrial transaminases was studied.

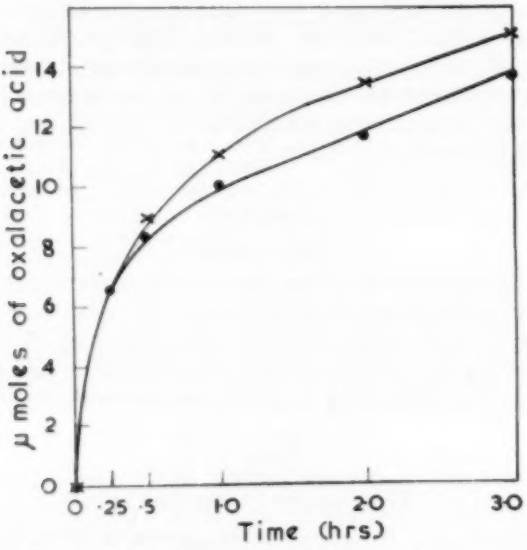

FIG. 1. Formation of oxaloacetate by transamination of aspartate and α-ketoglutarate. Mitochondria from 1-day-old seedlings (×); mitochondria from 2-day-old seedlings (●). Reaction system contained: 40 μmol. potassium L-aspartate; 40 μmol. potassium α-ketoglutarate; 100 μmol. phosphate buffer, pH 7·4 and 0·5 ml. mitochondrial suspension. Total volume 2·0 ml. Reaction stopped by the addition of 0·5 ml. aluminium sulphate reagent.

The coenzyme was found to increase the aspartate-glutamate transaminase activity by 12 per cent. and that of alanine-glutamate transaminase by 52 per cent., while the transaminases for glycine-glutamate, leucine-glutamate, ornithine-glutamate, and valine-glutamate were not affected.

Specificity of transaminases. To test the specificity of the transaminases, the rates of transamination of two amino-acids with α-ketoglutarate were assayed separately and combined. If two different enzymes are involved the combined rate of glutamate formation will be the sum of the two separate rates. From Table II, it can be seen that alanine-glutamate transaminase was not affected by aspartate, leucine, or valine. Similarly the aspartate-glutamate transaminase was unaffected by alanine but it was stimulated by leucine and valine. These latter differences can probably be explained in terms of the equilibrium. Leucine and valine can catalyse the degradation of oxaloacetic acid (Bessman

and Layne, 1950) and so the aspartate-glutamate transaminase reaction may be stimulated beyond the normal rate.

TABLE II

Amino-acid specificity of glutamate transaminases in mitochondria from 2-day-old seedlings

Amino-acids added	Relative activity of transaminases
L-aspartate	100
L-alanine	12
L-aspartate and L-alanine	112
L-leucine	2
L-aspartate and L-leucine	118
L-alanine and L-leucine	14
L-valine	0
L-aspartate and L-valine	117
L-alanine and L-valine	12

Conditions as in Table I except 80 μmol. of potassium α-ketoglutarate added. Duration of experiment 1 hour. Glutamic acid was estimated.

Reversibility. The reversibility of the alanine-glutamate and aspartate-glutamate reactions was demonstrated by the formation of alanine from pyruvate and glutamate and aspartate from oxaloacetate and glutamate. Amino-acids were identified by one-dimensional paper chromatography with the solvents of 80 per cent. phenol and *n*-butanol-acetic acid-water (4:1:5). In the aspartate-glutamate transaminase reaction, the reverse reaction was found to be about twice as fast as the forward reaction, even under conditions where the oxaloacetate concentration used in the reverse reaction was reduced to one-tenth of that of the other reactants. Using a soluble extract of mitochondria, the system aspartate plus α-ketoglutarate (40 μmol. each) gave an optical density change of 0·097 in 10 minutes at 280 mμ (λ max. of oxaloacetic acid) (Green *et al.*, 1945) and the reverse reaction, oxaloacetate and glutamate (4 and 40 μmol. respectively) gave an optical density change of 0·222 at 280 mμ in 10 minutes.

Oxidation of Amino-acids

Mitochondria prepared from 1- or 2-day seedlings, whether intact or broken, were found to be incapable of oxidizing the amino-acids L-alanine, glycine, and L-leucine. As these amino-acids can take part in transaminase reactions, the effect of α-ketoglutarate on their possible oxidation was studied, but in no case was oxygen uptake stimulated. Although oxygen uptake was not observed, some ammonia was released (Table III). Deamination of glycine and ornithine was enhanced slightly by α-ketoglutarate, while in the case of alanine and aspartate a marked stimulation was obtained.

The manner of aspartic acid breakdown was different from that of the other amino-acids as there proved to be two pathways dependent upon the age of

the seedlings from which the mitochondria were isolated. Mitochondria from seedlings up to 1 day old were capable of metabolizing aspartate alone, with associated oxygen uptake. Mitochondria from 2- to 4-day-old seedlings could not oxidize aspartate; extensive degradation of the amino-acid only occurred after the addition of organic acids. The detailed manner of operation of the two different degradation pathways will be considered separately.

TABLE III

Deamination of amino-acids by mitochondria from 2-day-old seedlings

	Ammonia formed (μm.)		
	None	α-ketoglutarate	Difference due to α-ketoglutarate
L-alanine	0·4	1·2	0·8
L-aspartate	0·8	7·4	6·6
L-glutamate	0·8	—	—
Glycine	0	0·2	0·2
L-leucine	0·2	0·2	0
L-ornithine	0·2	0·4	0·2

Reaction mixture contained: 40 μmol. of amino-acid; 40 μmol. of potassium α-ketoglutarate; 100 μmol. phosphate buffer, pH 7·4; 10 μmol. disodium EDTA; 20 μmol. magnesium sulphate; 1 μmol. ATP; 400 μmol. sucrose and 0·5 ml. of mitochondrial suspension. Total volume 2·0 ml. Duration of experiment 1 hour. Reaction stopped by the addition 0·3 ml. of 2N HCl.

One-day mitochondria. Aspartate or α-ketoglutarate are easily oxidized by mitochondria from 1-day-old seedlings (Fig. 2). When aspartate and α-ketoglutarate were supplied together to mitochondria the rate of oxygen uptake was not equal to the sum of their separate oxidation rates; an interaction of the two systems is therefore indicated. In contrast carbon dioxide production was not affected by combination of the two substrates (Fig. 3). Paper chromatography of extracts of mitochondria which were metabolizing aspartate showed the presence of α-alanine and glutamate. The amino-acids probably originated from pyruvate and α-ketoglutarate present in the mitochondria (see earlier) by transamination with aspartate. Since oxaloacetate is being produced by the aspartate-glutamate transaminase, pyruvate, and α-ketoglutarate would then be synthesized via the Krebs cycle reactions which are known to be operative in these mitochondria (Millerd *et al.*, 1951). The 1-day mitochondrial preparations were found to contain oxaloacetate decarboxylase and so carbon dioxide production could be due to the coupling of aspartate-glutamate transaminase with the decarboxylase.

Two-day mitochondria. These mitochondria could not oxidize aspartate alone. They were also incapable of affecting any decarboxylation of the amino-acid unless α-ketoglutarate was added (Fig. 4). The latter observation suggested that transamination occurred between aspartate and α-ketoglutarate to yield oxaloacetate which was then partially decarboxylated by an active oxaloacetate decarboxylase (30 per cent. reaction in 1 hour) present in these mitochondria.

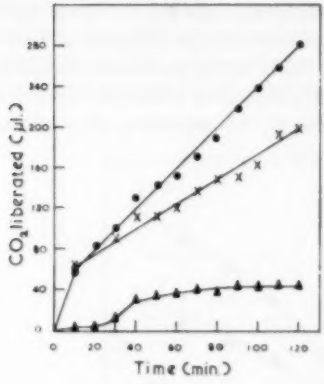

FIG. 3.

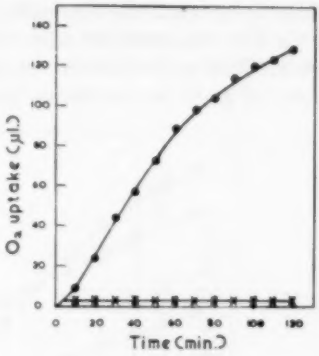

FIG. 5.

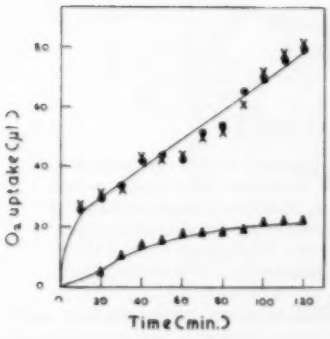

FIG. 2.

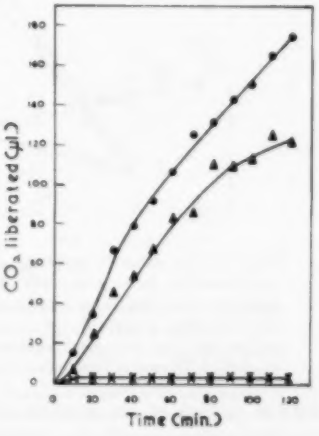

FIG. 4.

FIG. 2 (*bottom left*). The effect of aspartate on the oxidation of α-ketoglutarate by mitochondria from 1-day-old seedlings. Aspartate (●); aspartate and α-ketoglutarate (×); α-ketoglutarate (▲).

Readings corrected for endogenous. System contained: 40 µmol. potassium L-aspartate; 40 µmol. potassium α-ketoglutarate; 100 µmol. phosphate buffer, pH 7·4; 400 µmol. sucrose; 20 µmol. $MgSO_4$; 10 µmol. EDTA; 1 µmol. ATP and 0·5 ml. mitochondrial suspension. Total volume 2·0 ml.

FIG. 3 (*top left*). Decarboxylation of aspartate and α-ketoglutarate by mitochondria from 1-day-old seedlings. α-ketoglutarate and aspartate (●); aspartate (×); α-ketoglutarate (▲). Conditions as in Fig. 2.

FIG. 4 (*bottom right*). Decarboxylation of aspartate and α-ketoglutarate by mitochondria from 2-day-old seedlings. α-ketoglutarate and aspartate (●); aspartate (×); α-ketoglutarate (▲). Conditions as in Fig. 2.

FIG. 5 (*top right*). The effect of aspartate on the oxidation of α-ketoglutarate by mitochondria from 2-day-old seedlings. Aspartate or α-ketoglutarate and aspartate (×); α-ketoglutarate (●). Conditions as in Fig. 2.

A further feature of the 2-day mitochondria was that their normal capacity to oxidize α-ketoglutarate was markedly inhibited by the presence of aspartate (Fig. 5). The oxidation of other Krebs cycle acids was also inhibited by aspartate. Citric acid oxidation was partially inhibited whilst the oxidation of succinate (Fig. 6) was inhibited progressively by aspartate after an initial

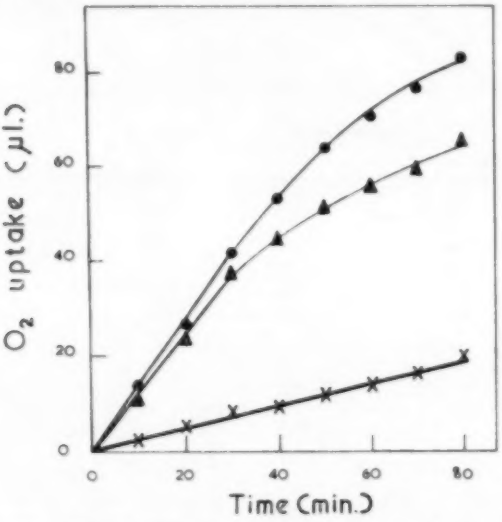

FIG. 6. The effect of aspartate and oxaloacetate on succinic oxidase of mitochondria from 2-day-old seedlings. Succinate (●); succinate and aspartate (▲); succinate and oxaloacetate, or oxaloacetate or endogenous (×). Systems contained: 40 µmol. of substrates; 100 µmol. phosphate buffer, pH 7·4; 400 µmol. sucrose; 20 µmol. $MgSO_4$; 10 µmol. EDTA; 1 µmol. ATP and 0·5 ml. mitochondrial suspension. Total volume 2·0 ml.

incubation period of 30 minutes. The oxidation of each organic acid was completely and immediately inhibited upon addition of oxaloacetate. It would then appear that the inhibition caused by aspartate was probably due largely to a secondary formation of oxaloacetate by a transamination process between aspartate and α-ketoglutarate. A comparable inhibition of the succinoxidase activity of liver homogenates by oxaloacetate was reported by Pardee and Potter in 1948, and oxaloacetate was also found to inhibit succinoxidase activity of particle preparations from avocado pear fruit (Avron and Biale, 1957) and bean seedlings (Beaudreau and Remmert, 1955). A more detailed experiment was performed to check the above reasoning. Aspartate and oxaloacetate were tested separately as inhibitors for the mitochondrial system oxidizing a mixture of α-ketoglutarate and succinate. The results are shown in Fig. 7. Addition of oxaloacetate to mitochondria oxidizing α-ketoglutarate and succinate at zero time, or at 30 minutes, completely inhibited oxygen uptake. Further, when oxaloacetate and α-ketoglutarate were

added together at zero time, sufficient oxaloacetate remained after 30 minutes to prevent the oxidation of added succinate. Aspartate required a 10-minute incubation period before α-ketoglutarate and succinate oxidation was inhibited completely. The reaction mixture containing aspartate, α-keto-

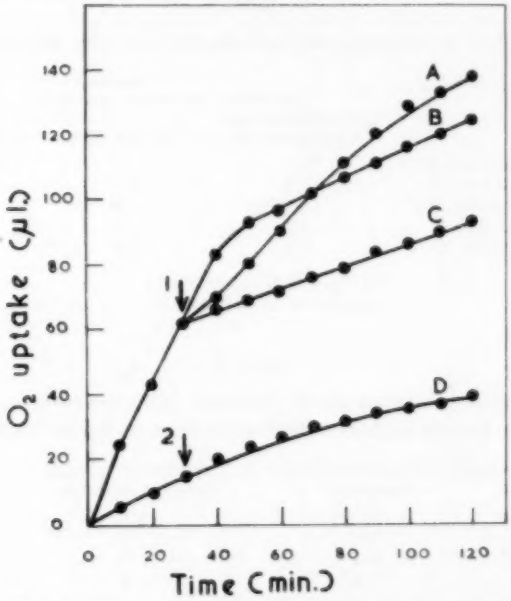

FIG. 7. The effect of aspartate and oxaloacetate on the oxidation of succinate and α-ketoglutarate by mitochondria from 2-day-old seedlings. A, α-ketoglutarate and succinate, at arrow 1 water added. B, α-ketoglutarate and succinate, at arrow 1 aspartate added. C, α-ketoglutarate and succinate, at arrow 1 oxaloacetate added. D, endogenous, or aspartate or oxaloacetate, or α-ketoglutarate, aspartate and succinate, or α-ketoglutarate, oxaloacetate and succinate, or α-ketoglutarate and aspartate, at arrow 2 succinate added or α-ketoglutarate and oxaloacetate, at arrow 2 succinate added. Reaction concentrations same as in Fig. 6.

glutarate and mitochondria was analysed after 1 hour, changes in oxaloacetate, malate, glutamate, aspartate, and oxygen uptake being measured. Glutamate and malate were found to be the main products together with a small amount of oxaloacetate (Table IV). The addition of a non-ionic detergent, O.P.C. 45, to the system disrupted the mitochondria and under these conditions, the two main products were oxaloacetate and glutamate, obviously produced by transamination. The structural integrity of the mitochondria was essential for the process of reducing oxaloacetic acid to malate and factors affecting mitochondrial stability (e.g. osmotic pressure of suspending medium; detergents) produced altered oxaloacetate/malate ratios (Table V). More oxaloacetate was formed from aspartate plus α-ketoglutarate as the sucrose

concentration of the medium was decreased and when mitochondria were disrupted completely by treatment with O.P.C. 45, maximal amounts of oxaloacetate were formed.

TABLE IV

Oxidation of L-*aspartate by mitochondria of 2-day-old seedlings*

	Systems		
	L-aspartate and α-ketoglutarate	α-ketoglutarate	L-aspartate and α-ketoglutarate*
Oxaloacetate formed (μm.)	1·1	0	5·3
L-malate formed (μm.)	5·5	0·2	0
L-glutamate formed (μm.)	6·1	0	9·4
L-aspartate used (μm.)	9·9	0	9·5
Oxygen uptake (μm.)	0	1·6	0

*0·1% O.P.C. 45 present.
Conditions as in Table III.

TABLE V

Effect of sucrose concentration on the formation of oxaloacetate from L-*aspartate and α-ketoglutarate in mitochondria from 2-day-old seedlings*

Sucrose concentration (molarity)	Oxaloacetate formed (μm./30 minutes)
0·06	4·1
0·2	3·2
0·4	2·8
0·4*	4·4

*0·1% O.P.C. 45 present.
The reaction system contained: 40 μmol. potassium L-aspartate; 40 μmol. potassium α-ketoglutarate; 100 μmol. phosphate buffer, pH 7·4 and 0·5 ml. mitochondrial suspension. Total volume 2·0 ml. Reaction stopped by the addition of 0·5 ml. aluminium sulphate reagent.

Evidence is provided by the data in Table IV that the reduction of oxaloacetate to malate in intact mitochondria is probably coupled to the oxidation of glutamic acid to α-ketoglutarate; this reflects itself in the lower amounts of glutamic acid apparently formed when malate was being synthesized. Davies (1953) has shown that pea mitochondria metabolizing oxaloacetate form malate by the coupling of DPN dependent dehydrogenases. Further experiments with soluble extracts from mung bean mitochondria of both 1- and 2-day-old seedlings indicated that such an interaction did occur between glutamic dehydrogenase and malic dehydrogenase (Fig. 8). The soluble extracts were obtained by treating mitochondria with an 0·1 per cent. solution of O.P.C. 45 and removing the mitochondrial fragments by centrifugation for 30 minutes at 20,000 g. Addition of oxaloacetate resulted in the rapid oxidation of DPNH. Simultaneous measurement of light absorption at 280 mμ, which is the λ max. for oxaloacetate, showed a marked decrease in E_{280}, suggesting that oxaloacetate

was reduced to malate. The favoured formation of malate by mitochondria from 2-day-old seedlings would account for the difference in the rates of deamination of glutamate when glutamate alone is being oxidized and when aspartate plus α-ketoglutarate are being oxidized (Table III).

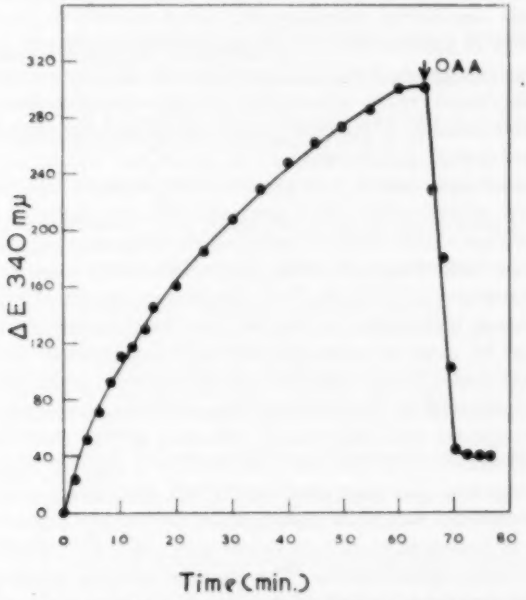

FIG. 8. Glutamic acid dehydrogenase and malic acid dehydrogenase of mitochondria. Reaction mixture contained: 60 μmol. potassium L-glutamate; 150 μmol. phosphate buffer, pH 7·4; 0·3 μmol. DPN and 0·5 ml. extract. At arrow, 2 μmol. of oxaloacetate added. Total volume 3·0 ml.

Aspartase appears to be absent from mitochondria of both 1- and 2-day-old seedlings, since no ammonia was produced when mitochondria suspended in water were incubated with aspartate.

DISCUSSION

The transaminases of bean mitochondria may be divided into two groups. The highly active aspartate-glutamate and alanine-glutamate transaminases form one group, whilst the other includes the slightly active transaminases for all other amino-acids. A similar classification may be applied to transaminases of other tissues (Meister, 1955). It is tempting to suggest that these striking differences of activity may be explained in terms of intracellular enzyme distributions. The aspartate-glutamate and alanine-glutamate transaminases may be predominantly mitochondrial enzymes. Such a distribution would

allow for the easy utilization of pyruvate and oxaloacetate produced by the activities of the mitochondrial enzymes catalysing the Krebs cycle reactions (Millerd *et al.*, 1951). The keto acid analogues of the other amino-acids are not concerned in the Krebs cycle and therefore may be produced at other sites within the cell. The appropriate transamination enzymes may also be located mainly outside the mitochondria.

Tables I and II indicate that the transaminations, aspartate-glutamate and alanine-glutamate, are probably catalysed by two different enzymes. Cruickshank and Isherwood (1958), who studied the aspartate-glutamate and alanine-glutamate transaminases in partially purified enzyme preparations from wheat germ, reached a similar conclusion.

Of the amino-acids tested, only aspartic and glutamic acids were oxidized by mung bean mitochondria. This contrasts with the ability of crude squash extracts to oxidize a wide range of amino-acids (Rogers, 1955). Mitochondria isolated from 1- and 2-day-old seedlings contained the same set of enzymes involved in aspartate metabolism, including the system for oxidizing DPNH. The difference in metabolism at the two ages would seem to be related to a reorganization of these enzymes within the mitochondria during the day interval. In the case of 2-day mitochondria the enzyme organization was such that DPNH produced by the dehydrogenase systems was largely oxidized by malic dehydrogenase and oxaloacetate, whereas in the 1-day mitochondria substantial amounts of DPNH were oxidized by a DPNH oxidase although malic dehydrogenase also competed for DPNH. Degradation of aspartate by the latter mechanism would be accompanied by oxygen uptake, whereas no transference of hydrogen to oxygen would occur in the sequence of reactions operating in 2-day mitochondria. The changed enzyme organization of the mitochondria may be the result of damage during the preparation of mitochondria. But it is also possible to view the change as a mechanism employed by the cell to convert oxaloacetate into a more stable form, malate, which could be accumulated in the vacuoles of plant cells (James, 1957) and re-enter their metabolism when a requirement for oxaloacetate existed.

ACKNOWLEDGEMENTS

This work was carried out while one of us (D. H. B.) received a maintenance grant from the Department of Scientific and Industrial Research. Roche Limited (England) kindly supplied pyridoxal-5′-phosphate.

LITERATURE CITED

Albaum, H. G., and Cohen, P. P. (1943). Transamination and protein synthesis in germinating oat seedlings. *J. Biol. Chem.* **149**, 19.

Avron, M., and Biale, J. B. (1957). Metabolic processes in cytoplasmic particles of the avocado fruit. V. Effect of oxalacetate on the oxidation of pyruvate and succinate. Ibid. **225**, 699.

Beaudreau, G. S., and Remmert, L. F. (1955). Krebs cycle activity of particles from bean seedlings. Ibid. **55**, 469.

BESSMAN, S. P., and LAYNE, E. C. (1950). The stimulation of the non-enzymatic decarboxylation of oxalacetic acid by amino-acids. *Arch. Biochem.* **26,** 25.

BONE, D. H. (1959). The metabolism of citrulline and ornithine in mung bean mitochondria. *Plant Physiol.* (In the press.)

BRAUNSTEIN, A. E., and BYCHKOV, S. M. (1939). A cell-free enzymatic model of L-amino acid dehydrogenase (l-deaminase). *Nature,* **144,** 751.

—— and KRITZMANN, M. G. (1937). Über den Ab- und Aufban von Aminosauren durch Umaninierung. *Enzymologia,* **2,** 129.

CRUICKSHANK, D. H., and ISHERWOOD, F. A. (1958). Glutamic-alanine and glutamic-aspartate transaminases of wheat germ. *Biochem. J.* **69,** 189.

DAMODARAN, M., and NAIR, K. R. (1938). Glutamic acid dehydrogenase from germinating seeds. Ibid. **32,** 1064.

DAVIES, D. D. (1953). The Krebs cycle enzyme system of pea seedlings. *J. Exp. Bot.* **4,** 173.

—— (1956). Soluble enzymes from pea mitochondria. Ibid. **7,** 203.

GREEN, D. E., LELOIR, L. F., and NOCITO, V. (1945). Transaminases. *J. Biol. Chem.* **161,** 559.

JAMES, W. O. (1957). Reaction paths in the respiration of higher plants. *Advances in Enzymol.* **18,** 281.

KREBS, H. A., and EGGLESTON, L. V. (1945). Metabolism of acetoacetate in animal tissues. I. *Biochem. J.* **39,** 408.

LEONARD, M. J. K., and BURRIS, R. H. (1947). A survey of transaminases in plants. *J. Biol. Chem.* **170,** 701.

MEISTER, A. (1955). Transamination. *Advances in Enzymol.* **16,** 185.

—— SOBER, H. A., and TICE, S. V. (1951). Determination of aspartic and glutamic acids by enzymatic decarboxylation. *J. Biol. Chem.* **189,** 591.

MILLERD, A., BONNER, J., AXELROD, B., and BANDURSKI, R. S. (1951). Oxidative and phosphorylative activity of plant mitochondria. *Proc. Nat. Acad. Sci. U.S.* **37,** 855.

NOSSAL, P. (1952). Estimation of L-malate and fumarate by malic decarboxylase of *Lactobacillus arabinosus*. *Biochem. J.* **50,** 349.

PARDEE, A. B., and POTTER, V. R. (1948). Inhibition of succinic dehydrogenase by oxalacetate. *J. Biol. Chem.* **176,** 1085.

RAUTANEN, N. (1946). Transamination in green plants. Ibid. **163,** 687.

—— and TAGER, J. M. (1955). Oxidation of amino-acids by plant mitochondria. *Ann. Acad. Sci. Fennica. A. II,* **60,** 241.

ROGERS, B. J. (1955). Oxidation and decarboxylation of amino-acids by squash preparations. *Plant Physiol.* **30,** 186.

VIRTANEN, A. I., and LAINE, T. (1938). Biological synthesis of amino-acids from atmospheric nitrogen. *Nature,* **141,** 748.

WILSON, D. G., KING, K. W., and BURRIS, R. H. (1954). Transamination in plants. *J. Biol. Chem.* **208,** 863.

Changes in Enzyme Activity of Fungi during Nitrogen Starvation

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SUMMARY

The intracellular proteinase enzymes of *Penicillium griseofulvum* and other fungi increase to levels several times higher than that in growing mycelium in the first few hours of nitrogen starvation, and remain at the higher level unless more assimilable nitrogen is supplied. The rise in proteinase does not take place in absence of oxygen or in the presence of *p*-fluorophenylalanine, and is not accompanied by net protein breakdown in the mycelium unless synthetic processes are inhibited by lack of oxygen or exogenous carbohydrate.

The proteinase activity changes during the life cycle of the fungus, being absent in the spores, very low in the early stages of germination, and tending to rise steadily during growth. In some conditions the induction of sporulation is associated with a rise in proteinase, but there is no simple relation between the two processes. The results are considered to support the view that intracellular proteinase plays a part in protein turnover in the fungal cell.

INTRODUCTION

In several species of *Penicillium* it has been found (Morton, England, and Towler, 1958) that an important condition for the occurrence of asexual sporulation is the absence of assimilable nitrogen. Sporulation may be induced, in certain conditions, simply by depriving the fungus of nitrogen, and the induction of sporulation may then be completely reversed by adding a fresh supply of nitrogen in the early stages of the process. The association between nitrogen starvation and the induction of sporulation suggested that a study of the enzymatic changes in the fungus when deprived of nitrogen might throw light on the nature of the inductive process. Attention was directed in the first place to the activity of the intracellular proteolytic enzymes. The activity of these enzymes was found to change markedly in nitrogen starvation. This paper gives some account of these enzymatic changes and discusses their significance in relation to morphogenesis.

MATERIAL AND METHODS

Except where other species are specially mentioned, the experiments were done with *Penicillium griseofulvum* Dierckx. The fungus was grown in submerged culture on a shaker in a standard nitrate medium with 5 per cent. glucose. The composition of the medium and general conditions of culture were as previously described (Morton, England, and Towler, 1958). In these cultural conditions *P. griseofulvum* remains vegetative and does not sporulate

even after all the nitrogen in the medium is exhausted. If, however, the young rapidly growing mycelium, about 18–24 hours after inoculation, is transferred to a nitrogen-free glucose medium practically every individual mycelium (thallus) sporulates 10–12 hours after transfer. The nitrogen-free medium contains all the components of the full medium (5 per cent. glucose, salts, trace elements) with the exception of any source of nitrogen (supplied in the complete medium as potassium nitrate). The transfer of mycelium to nitrogen-free glucose medium thus provides a convenient experimental system for the study of induction of sporulation. This system was used in the experiments to be described.

Intracellular proteinase activity was determined in samples of fresh mycelium, frozen rapidly by means of solid CO_2, and then allowed to autolyse at 25° C. (the temperature of culture) in a small volume of phosphate buffer at pH 7·0 for a standard time (usually 4 hours). The increase in free amino-nitrogen over the initial level in a frozen unautolysed replicate sample was determined as ammonia liberated by the action of ninhydrin (MacFadyen, 1944) and estimated by Nessler's reagent after vacuum distillation (Archibald, 1943). The increase in free amino-nitrogen was taken as a measure of the activity of the intracellular proteinases. Preliminary tests showed that the proteinase activity of frozen samples stored at −10° C. was remarkably stable. Replicate samples of a single batch of frozen mycelium showed no change when proteinase activity was determined at intervals over a period of 6 weeks. The rate of increase in free amino-nitrogen was found to be linear for 10–12 hours in the test conditions, and may therefore be reasonably taken as a measure of the intracellular proteinase activity. This relatively simple procedure for determining intracellular proteinase activity has several advantages. Experimental samples may be kept for a few days and estimated in one batch. Enzyme activity is measured in conditions entailing a minimum of interference with cell structure, since the material is not ground up, and only a small volume of buffer is added, at a pH close to the normal internal pH of the fungus. Finally, enzyme activity is measured on what are presumably the natural cellular substrates.

It will be appreciated that what is measured is the activity of a group of intracellular proteolytic enzymes, about whose specificities nothing can yet be said. For convenience, however, this activity is referred to simply as the 'proteinase' activity of the mycelium. Throughout this paper proteinase activity is expressed as free amino-nitrogen formed per hour as per cent. total nitrogen of the mycelium. This is practically equivalent to per cent. total nitrogen hydrolysed per hour.

EXPERIMENTAL RESULTS

1. *Proteinase in nitrogen-starved mycelium*

When growing mycelium of *Penicillium griseofulvum* is transferred from complete to nitrogen-free medium a rapid rise in proteinase activity takes place within the first few hours after transfer. The results of a typical experiment

are shown in Fig. 1. The mycelium in complete medium shows a steady level of proteinase activity, although rising somewhat towards the end of the growth period (as is discussed later). The physical disturbance of transfer of mycelium to nitrogen-free medium does not cause any immediate change in proteinase activity, but following transfer a rapid rise begins. In 4 hours there is an 8-fold increase in proteinase activity which then remains steady at the enhanced level.

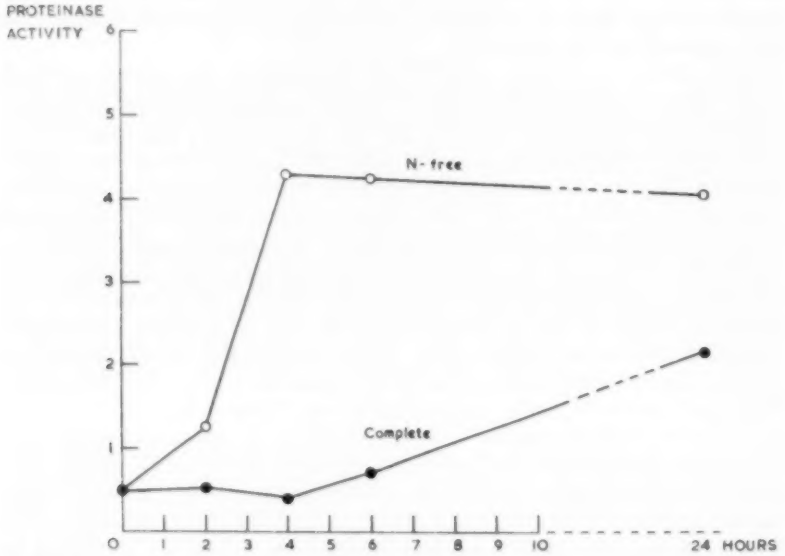

FIG. 1. Internal proteinase activity of *Penicillium griseofulvum* in complete and in nitrogen-free medium.

The general course of proteinase activity in nitrogen-starved mycelium is further illustrated by the results of several experiments represented in Fig. 2. In each case the effect of transferring mycelium to nitrogen-free medium is to induce a rapid rise in intracellular proteinase activity, which increases 5–10 times in 4–6 hours, after which it remains steady or tends to fall again, but does not fall to the initial level. This striking rise in proteinase activity of the fungus takes place in the same conditions and during the same period of time as does the induction of sporulation (experiments to be reported in a separate publication). Induction can be reversed and sporulation prevented by the addition of assimilable nitrogen during this period. It was therefore of interest to examine the effect of added nitrogen on the change in proteinase activity. In Fig. 3 the result of an experiment is shown, in which potassium nitrate was added 4 hours after transfer of mycelium to nitrogen-free medium. The addition of nitrate caused a sharp fall in proteinase activity but did not reduce it to

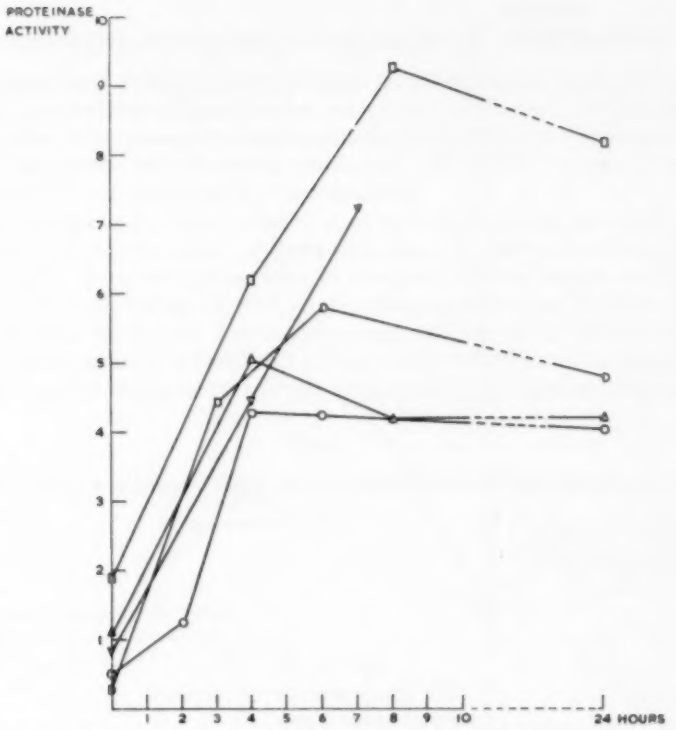

FIG. 2. Internal proteinase activity of *Penicillium griseofulvum*, in nitrogen-free medium.

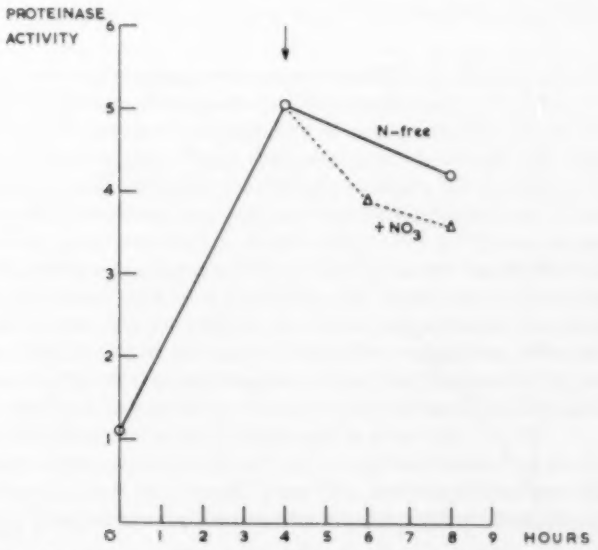

FIG. 3. Internal proteinase activity of *Penicillium griseofulvum* in nitrogen-free medium. Effect of adding fresh nitrate.

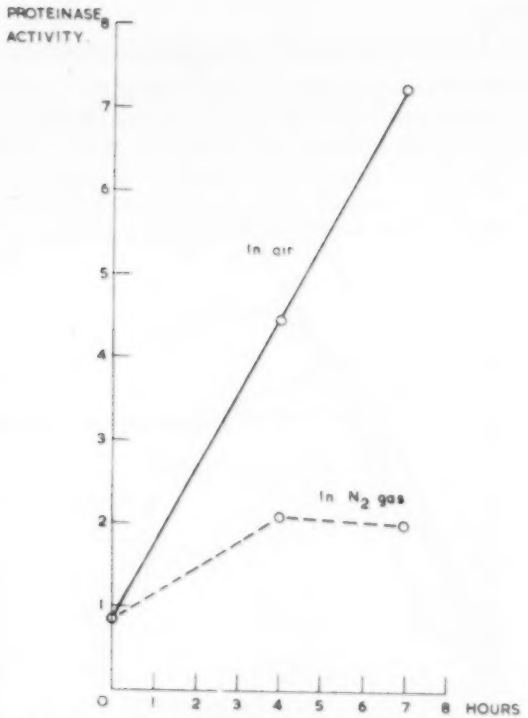

FIG. 4. Internal proteinase activity of *Penicillium griseofulvum* in air and nitrogen.

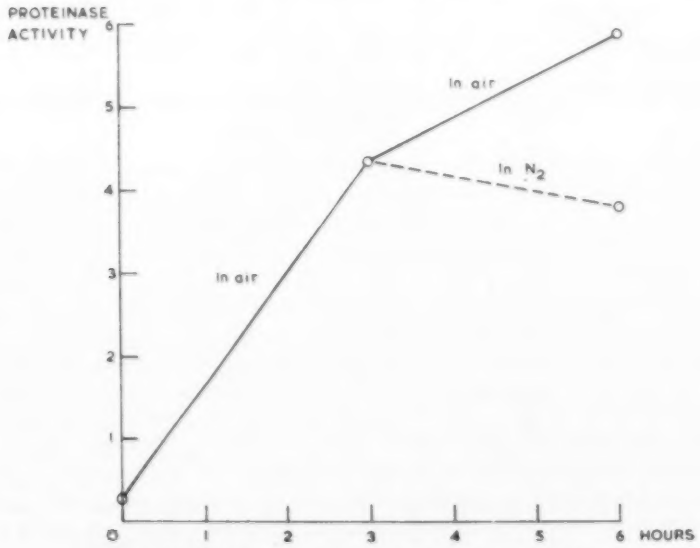

FIG. 5. Internal proteinase activity of *Penicillium griseofulvum*.

the original level. It appears that in a large part of the mycelium the enhanced level of the proteinase activity persists when once it is attained. The fall due to the addition of nitrate can be largely, although not completely, accounted for by the assumption that the newly synthesized cell material has proteinase at the original level characteristic of growing cells.

The increase in proteinase activity in nitrogen-free conditions may be due to several causes—increased synthesis of enzyme, formation of active enzyme from inactive precursor, breakdown of a barrier between enzyme and substrate in the cell, change in state of the cell proteins rendering them more susceptible to hydrolysis. Experiments were carried out in an attempt to decide between these possibilities. It was found that the rise in proteinase activity does not take place in anaerobic conditions. In the experiment recorded

TABLE I

Effect of p-fluorophenylalanine on proteinase in Penicillium griseofulvum

		Proteinase activity of mycelium (as % total N hydrolysed per hour)
Mycelium immediately after transfer to N-free medium	..	1·07
Mycelium after 5 hours in N-free medium	No addition	3·97
		3·77*
" "	$+10^{-3}$M *p*-fluorophenylalanine	0·30
" "	$+10^{-4}$M *p*-fluorophenylalanine	2.42

* Proteinase test in presence of 10^{-3}M *p*-fluorophenylalanine

in Fig. 4 air was replaced by nitrogen gas immediately after the mycelium was transferred to nitrogen-free medium. The small initial rise in proteinase of the mycelium in nitrogen gas probably occurred during the period before anaerobiosis was complete. When anaerobic conditions were fully established there was no rise in proteinase, possibly even a slight fall. A similar result was observed when mycelium was put into anaerobic conditions 3 hours after transfer to nitrogen-free medium, as recorded in Fig. 5. The rise in proteinase ceases in the absence of oxygen and is replaced by a slow but distinct fall. If the change in proteinase activity depended on the breakdown of a metabolically maintained barrier between enzyme and substrate, it would be expected that proteinase activity would increase in anaerobic conditions, whereas in fact it decreases slowly. The results suggest rather that the increase in proteinase activity in nitrogen-free medium represents a synthesis of additional enzyme, for which the energy of aerobic respiration is required.

In further experiments the effect of *p*-fluorophenylalanine on the fungus in nitrogen-free medium was tested, since this compound has been shown to inhibit the formation of adaptive enzymes in yeast (Halvorson and Spiegelman,

1952) and in bacteria (Cohen and Munier, 1959). The results with two concentrations of *p*-fluorophenylalanine are given in Table I. At the higher concentration *p*-fluorophenylalanine completely prevents the usual rise in proteinase activity, although it clearly has no effect on the activity of enzyme already formed, if added during test. The behaviour of the system in anaerobic conditions, together with the experiments with *p*-fluorophenylalanine, thus provide strong evidence that the rise in proteinase activity on transfer to nitrogen-free medium is due to the synthesis of new enzyme. Both in anaerobic conditions and in presence of inhibitory concentrations of *p*-fluorophenylalanine a slow fall in proteinase activity has been consistently observed. This suggests that in living mycelium in normal aerobic conditions proteinase is possibly being synthesized and broken down simultaneously. Whether this is so or not, it seems clear that proteinase is very actively synthesized by the fungus in the first few hours of nitrogen starvation.

Determinations of free amino-nitrogen in the mycelium after transfer to nitrogen-free medium show that the level of this fraction does not change significantly. Thus in spite of the increase in intracellular proteinase there is no net protein breakdown or rise in soluble nitrogen in the mycelium. This agrees with morphological observations which show some growth in length and thickness of the mycelium in these conditions, accompanied by the differentiation of conidiophores and ultimately of spores. The absence of net protein breakdown may be due to the existence of a physical barrier between the cell proteins and the proteinase in the living cell. If there is no physical barrier, then the existence of simultaneous synthesis and breakdown of protein (turnover of protein) would be indicated.

In order to investigate this question the following experiment was done. Mycelium (24 hours old) was transferred to nitrogen-free medium and kept in aerobic conditions to promote the rise of intracellular proteinase. Three hours after transfer some of the mycelium was subjected to anaerobic conditions by passing in gaseous nitrogen, whilst the remaining mycelium continued in aerobic conditions. Replicate samples of mycelium of equal initial fresh weight and nitrogen content were submitted to experimental treatment, so that a nitrogen balance sheet could be determined at different times. The mycelium nitrogen was divided into fractions on the basis of solubility in dilute trichloracetic acid; the total nitrogen in each fraction was determined by semi-micro Kjeldahl. The insoluble nitrogen includes protein and nucleic acid nitrogen, whilst the soluble nitrogen includes nitrogen of the free amino-acids, nucleotides, and other metabolites. Nitrogen lost from mycelium to the medium was determined as a check, although these figures are liable to greater error owing to dilution by the relatively large volume of medium.

The principal results of this experiment are assembled in Table II. In the aerobic mycelium the usual rapid rise in proteinase induced in nitrogen-free medium continued for about 6 hours, falling slightly in the following 18 hours. The mycelium increased in dry weight and was sporing profusely at the end of the experiment. There was no increase in the level of soluble nitrogen

in the mycelium and no significant loss of nitrogen to the medium. The mycelium which was placed in anaerobic conditions after 3 hours showed no further rise in proteinase but instead a slow fall. The mycelium lost dry weight and did not sporulate. The nitrogen figures show that protein breakdown begins as soon as anaerobiosis is established. The rate of protein breakdown actually found is quite close to the rate deduced from the proteinase measurements.

TABLE II

Effect of aerobic and anaerobic conditions on Penicillium griseofulvum *in nitrogen starvation*

Sample	Proteinase (% Total N hydrolysed/hr.)	Mycelium-N (mg./sample) Insol.	Soluble	N lost to medium (mg./sample)
Initial (ex-complete medium)	0·3	20·6	1·4	..
3 hours Aerobic (1)	4·2	21·3	0·7	0·2
3 hours Aerobic (2)	4·6	20·7	0·7	0·5
6 hours Aerobic	5·8	19·6	0·9	0·3
6 hours Anaerobic	3·8	18·5	0·9	1·3
24 hours Aerobic	4·9	19·6	0·8	1·0
24 hours Anaerobic	2·6	11·5	0·4	10·1

The fact that protein breakdown begins as soon as the synthetic processes are arrested by the absence of oxygen is evidence against the existence in the normal cell of a barrier between the proteinase enzymes and their substrates. This evidence is strengthened by the observation recorded earlier, and confirmed in this experiment, that the rate of proteinase activity does not rise in anaerobic conditions as would be expected were enzyme activity normally prevented by a metabolically maintained barrier. In other experiments we have found that proteolysis begins at a very early stage in mycelium in which synthetic activity is limited by the absence of an external supply of carbohydrate. If the absence of a barrier is accepted, then our observations indicate the existence in the fungal cell of protein turnover, in which the intracellular proteinase plays an essential role. In nitrogen-starved mycelium the rate of turnover is about 6 per cent. of the total mycelial nitrogen per hour (mean of many observations with *Penicillium griseofulvum*). There is no evidence that the growing mycelium in complete medium behaves in any way differently from nitrogen-starved mycelium in relation to anaerobiosis. It may be concluded that protein turnover is also characteristic of growing mycelium, although the rate is lower, being about 1 per cent. of the total mycelial nitrogen per hour. Protein turnover of this order of magnitude has recently been demonstrated for fungi in this laboratory by more direct methods with the aid of isotopic nitrogen, and this evidence will be reported separately.

2. *Behaviour of other fungi*

Several other fungi were examined to see whether they respond to transfer to nitrogen-free conditions in the same way as *Penicillium griseofulvum*. The results given in Table III show that the growing mycelium of all the fungi tested responded to nitrogen-free conditions by a marked rise in intracellular proteinase; the phenomenon therefore appears to be a general one among fungi. The basic proteinase activity of the growing mycelium of all the fungi is of the same order as in *P. griseofulvum*, but exact comparison cannot be made as the total mycelial nitrogen was not determined in these experiments.

TABLE III

Change of proteinase activity of various fungi transferred to nitrogen-free conditions

Organism	Age (hours from inoculation) at transfer	% Glucose in N-free medium	Change in proteinase during 5 hours in nitrogen-free medium (X initial level)
Penicillium griseofulvum	24	5	3–15
		1	12·8
		10	12·6
P. chrysogenum	48	5	1·0
	24	1	7·0
		10	8·0
Scopulariopsis brevicaulis	48	1	1·7
		10	1·6
Aspergillus niger	24	1	3·1
		10	3·9
Gibberella fujikuroi	24	1	5·1
		10	7·0
	48	1	1·8
		10	1·0

The rise in proteinase in response to nitrogen starvation is not affected by rather wide variations in the glucose concentration of the medium. It will be seen, however, that there is evidence of a relation between response and the age of the mycelium at the time of transfer. In *Penicillium griseofulvum* and *Gibberella fujikuroi* 48-hour mycelium shows a much smaller relative rise in proteinase than does 24-hour mycelium. The relatively slight response of older mycelium has been repeatedly observed with *P. griseofulvum*. It may be connected with the fact that the basic proteinase level of 48-hour mycelium is generally higher than at 24 hours, and this in turn may be linked with the fact that the nitrogen in the medium is (in the conditions of these experiments) usually exhausted between 40 and 48 hours after inoculation. These considerations led us to investigate the proteinase activity of *P. griseofulvum* throughout its growth cycle.

3. *Changes in proteinase during development*

The proteinase activity of *Penicillium griseofulvum* was determined at intervals throughout the life cycle when the fungus was grown in shake culture in

complete nitrate medium in the usual experimental conditions. Special attention was given to observation of the precise time when the nitrogen in the medium became exhausted. The results are given in Table IV. Proteinase appears to be absent from the spores and remains very low up to 8 hours, by which time the spores have swollen, but not yet developed, germ tubes. In the next few hours the proteinase rises rapidly and at 12–16 hours the level

TABLE IV

Intracellular proteinase activity of Penicillium griseofulvum *during growth in nitrate medium*

Time from inoculation (hours)	Stage of development	Proteinase (% total N hydrolysed/hour)
0	Spores	0
8	Spores swollen, no germ tubes yet	0·10
12	Germ tubes	0·32
16	Short mycelia	0·27
20	Mycelial branching beginning	0·35
20–28	Mycelial, rapid growth	0·3–1·4 (Mean 1·0)
40	Nitrate still present but approaching exhaustion	2·1
42	Nitrate disappeared from medium. Glucose still present, mycelial dry weight continues to increase	2·2
44		3·5
46		5·0
60		5·0

comes within the range found in young mycelium in which growth is close to exponential. It may be noted that mycelium becomes capable of sporulation at about 12–16 hours but not earlier (see Morton, England, and Towler, 1958).

There is a distinct rise in proteinase in the later stages of growth whilst nitrogen is still present in the medium. Growth usually ceases to be exponential at this stage. Immediately following nitrogen exhaustion the proteinase rises rapidly, as in the transfer experiments with younger mycelium, although the relative increase is smaller. Older mycelium in which the proteinase level is already high owing to exhaustion of nitrogen in the medium shows no proteinase increase on transfer to fresh nitrogen-free medium.

In the experiments reported in Table IV the mycelium remained vegetative throughout the life cycle. The picture may be completed by reference to an experiment in which *Penicillium griseofulvum* was grown in complete nitrate medium with the normal carbon source, pure glucose, until the mycelium was about 24 hours old. Commercial (crude) glucose has been shown by previous work (Morton, England, and Towler, 1958) to induce abundant sporulation in *P. griseofulvum* even in presence of assimilable nitrogen. The culture in glucose-nitrate was therefore divided into two parts; to one part an extra 1 per cent. pure glucose was added, to the other 1 per cent. crude glucose. The proteinase activity of the mycelium was determined at intervals

with the results shown in Table V. During the induction of sporing in presence of crude glucose there is a significant rise in proteinase, although the final behaviour of the two cultures does not appear very different, the main rise in proteinase evidently depending on exhaustion of nitrogen from the medium.

TABLE V

Proteinase activity of Penicillium griseofulvum *in sporing and non-sporing conditions in nitrate medium*

Time from inoculation (hours)			Proteinase (as per cent. Total Nitrogen hydrolysed/hour) 24	28	32	48	Appearance of mycelium at 48 hours
1·4	+1%	pure glucose	1·4	1·2	7·2		Vegetative
1·4	+1%	*crude* glucose	2·2	2·5	6·1		Sporing profusely

DISCUSSION

The results which have been presented show that many fungi respond to nitrogen starvation by a rapid increase in intracellular proteinase, the consequence of an increased synthesis of the enzymes in question. Thus exhaustion of nitrogen in the medium does not lead to a condition of stability in the fungal cell, but rather to very rapid changes in the pattern of at least some intracellular enzymes. Whatever the mechanism by which this response to environmental change is accomplished, the increase in proteinase may perhaps be considered adaptive, in a broad sense, as enabling the organism to build new patterns of enzymes, permitting survival (frequently through some form of reproductive development) when assimilable nitrogen is absent from the external environment.

The observations provide evidence strongly supporting the view that continuous protein turnover is a basic feature of fungal metabolism, and that the intracellular proteinases play a significant part in the process. More direct evidence of protein turnover in fungi has recently been obtained in our laboratory and will be reported in a separate paper. In spite of some evidence to the contrary (e.g. Hogness, Cohn, and Monod, 1955; Koch and Levy, 1955) recent work (summarized by Urbá, 1959) has tended to confirm the existence of protein turnover both in growing and resting cells. Mention may be made of the work of Urbá (1959) and Pollock and Kramer (1958) and Mandelstam (1957, 1958) with bacteria, of Halvorson (1958 *a*, *b*) with yeast, and of Eagle, Piez, Fleischmann, and Oyama (1959) with mammalian cells. In bacteria and fungi the rate of turnover of protein has been found to be higher in resting than in growing cells. Our observations on proteinase activity are in line with these findings and indicate that a higher rate of

turnover may be a general characteristic of cells which have passed the phase of most active growth.

The absence of proteinase in the spores and the changes in proteinase during the growth cycle of *Penicillium*, especially the rise in activity in older mycelium before nitrogen exhaustion, suggest a relation between these enzymes and the processes of differentiation and ageing.

Finally, the possible connexion between proteinase changes and the induction of sporulation must be briefly considered. Experiments on the physiology of sporulation have been already described (Morton, England, and Towler, 1958) and an account of further work is in preparation. It is, however, only necessary to refer to these experiments in a general way here. There does not seem to be any simple causal relation between a rise in proteinase and the induction of sporulation, although the two processes occur simultaneously in *Penicillium griseofulvum* after transfer to nitrogen-free medium in the conditions described earlier. If, however, the glucose concentration in the nitrogen-free medium is lowered to 0·5—1 per cent. (instead of the usual 5 per cent.) sporulation is practically absent, but the proteinase actively increases in the usual way. The two processes are thus readily dissociated in *P. griseofulvum*; in other fungal species increased proteinase in nitrogen-starvation is not accompanied by sporulation. Certain substances which inhibit sporulation in *P. griseofulvum* without markedly affecting growth are without effect on the increase in proteinase in inductive conditions. On the other hand, a rise in proteinase is associated with several diverse conditions inducing sporulation in *P. griseofulvum*—nitrogen exhaustion, crude glucose, sub-aerial conditions—in such a way as to suggest that an increase in proteinase might be essentially connected with sporulation in some conditions. In the complex process of morphogenesis over-simple relations can scarcely be expected. It does seem, however, that the role of intracellular proteinases in the physiology of development is worth further investigation.

LITERATURE CITED

Archibald, R. M. (1943). The estimation of ammonia in the presence of glutamine. *J. Biol. Chem.* **151**, 141.

Cohen, G. N., and Munier, R. (1959). Effets des analogues structuraux d'aminoacides sur la croissance, la synthèse de proteines, et la synthèse d'enzymes chez *Escherichia coli*. *Biochim. Biophys. Acta*, **31**, 347.

Eagle, H., Piez, K. A., Fleischmann, R., and Oyama, V. I. (1959). Protein turnover in mammalian culture cells. *J. Biol. Chem.* **234**, 592.

Halvorson, H. (1958*a*). Intracellular protein and nucleic acid turnover in resting yeast cells. *Biochim. Biophys. Acta*, **27**, 255.

—— (1958*b*). Studies on protein and nucleic acid turnover in growing cultures of yeast. Ibid. **27**, 267.

Halvorson, H. O., and Spiegelman, S. (1952). The inhibition of enzyme formation by amino acid analogues. *J. Bact.* **64**, 207.

Hogness, D. S., Cohn, M., and Monod, J. (1955). Studies on the induced synthesis of β-galactosidase in *Escherichia coli*: the kinetics and mechanism of sulfur incorporation. *Biochim. Biophys. Acta*, **16**, 99.

Koch, A. L., and Levy, H. R. (1955). Protein turnover in growing cultures of *Escherichia coli*. *J. Biol. Chem.* **217**, 947.

MacFadyen, D. A. (1944). The determination of ammonia evolved from α-amino acids by ninhydrin. *J. Biol. Chem.* **153,** 507.

Mandelstam, J. (1957). Turnover of protein in starved bacteria and its relationship to the induced synthesis of enzyme. *Nature,* **179,** 1179.

—— (1958). Turnover of protein in growing and non-growing populations of *Escherichia coli*. *Biochem. J.* **69,** 110.

Morton, A. G., England, D. J. F., and Towler, D. A. (1958). The physiology of sporulation in *Penicillium griseofulvum* Dierckx. *Trans. Brit. mycol. Soc.* **41,** 39.

Pollock, M. R., and Kramer, M. (1958). Intermediates in the biosynthesis of bacterial penicillinase. *Biochem. J.* **70,** 665.

Urbá, R. C. (1959). Protein breakdown in *Bacillus cereus*. Ibid. **71,** 513.

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MacFadyen, D. A. (1944). The determination of ammonia evolved from α-amino acids by ninhydrin. *J. Biol. Chem.* **153,** 507.

Mandelstam, J. (1957). Turnover of protein in starved bacteria and its relationship to the induced synthesis of enzyme. *Nature,* **179,** 1179.

—— (1958). Turnover of protein in growing and non-growing populations of *Escherichia coli*. *Biochem. J.* **69,** 110.

Morton, A. G., England, D. J. F., and Towler, D. A. (1958). The physiology of sporulation in *Penicillium griseofulvum* Dierckx. *Trans. Brit. mycol. Soc.* **41,** 39.

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